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A class of new synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) effectively induce apoptosis of prostate cancer cells. Our previous study demonstrated that AHPN/CD437-induced apoptosis of LNCaP prostate cancer cells requires the expression of TR3 (also called nur77 or NGFI-B) that is an orphan member of the steroid/thyroid/retinoid receptor superfamily and its nuclear export and mitochondrial localization. In studying how TR3 nuclear export, mitochondrial targeting and apoptosis induction in prostate cancer cells are regulated, we have demonstrated that the migration of TR3 from the nucleus to the cytoplasm requires retinoid X receptor (RXR) through their heterodimerization. In addition, we show that the TR3 cytoplasmic localization and apoptotic effect are inhibited by RXR ligand 9-cis retinoic acid. Moreover, we demonstrate that TR3 interacts with Bcl-2 and that the interaction is essential for TR3 to target mitochondria and to induce apoptosis. These data not only enhance our understanding the molecular mechanism by which TR3 nuclear export, mitochondrial targeting and apoptosis induction are regulated but also provide important information for developing novel strategies for inducing apoptosis of prostate cancer cells.

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INTRODUCTION

Prostate cancer is the second most common cause of male cancer death in the West. Conventional chemotherapy and radiotherapy are still of limited effectiveness. Recent progress has suggested that induction of cancer cell death is a plausible way to restrict tumor growth, and many chemotherapeutic drugs induce death of cancer cell. Retinoids, vitamin A and its analogs, are known to induce death of prostate cancer cells. However, the effectiveness of conventional vitamin A derivatives, such as all-*trans* retinoic acid, is limited to androgen-dependent prostate cancer cells. Recently, a new class of synthetic vitamin A derivatives related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) have been found to effectively induce death of both androgen dependent and -independent prostate cancer cells (1-3), suggesting that they represent a new class of chemotherapeutic agents for treating advanced hormone refractory prostate cancer. We subsequently showed that TR3/nur77, an orphan member of the steroid/thyroid/retinoid receptor superfamily, is required for induction of death of both androgen-dependent and -independent prostate cancer cells by AHPN/CD437 and other death-inducing agents (3). Moreover, we discovered that TR3/nur77, in response to AHPN/CD437 and other apoptotic stimuli, migrated from the nucleus to the cytoplasm, where it targeted mitochondria, resulting in cytochrome c release and apoptosis (3).

This application focuses on the molecular mechanism by which TR3-mediated apoptosis of prostate cancer cells is mediated and regulated. In the proposed studies, we plan to investigate whether this novel nuclear-to-mitochondrial pathway for apoptosis can be extended to other members of the TR3/nur77 family and to other prostate cancer cell lines, especially the hormone-refractory prostate cancer cells. In addition, we will study the mechanism by which translocation of TR3/nur77 from the nucleus to cytoplasm is regulated. Furthermore, we will study physical interactions between TR3/nur77 and members of the Bcl-2 family. Results from these studies will enhance our understanding of the mechanism by which TR3/nur77 induces apoptosis of androgen-dependent and -independent prostate cancer cells and of the molecular control of prostate cancer growth. They should provide a molecular basis for the identification of agents that induce association of TR3/nur77 with mitochondria, resulting in possible conversion of TR3/nur77 from a growth promoting to death inducing molecule in prostate cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

In the past funding year, we conducted various experiments to address the specific aims proposed in the grant application as described below.

1. Regulation of TR3 nuclear export, mitochondrial targeting and apoptosis by RXR and its ligands

TR3 is known to heterodimerize with RXR (4,5). Several experiments were conducted to determine the effect of RXR and its ligands on TR3 nuclear export, mitochondrial localization and its induced apoptosis. In LNCaP cells, RXR was found exclusively in the nucleus in the absence of treatment. However, when cells were treated with apoptotic stimulus TPA, RXR translocated to the cytoplasm, where it was colocalized with mitochondria (Figure 1A).

Pretreatment of cells with RXR agonists 9-*cis* RA or SR11237 prevented RXR mitochondrial localization. The localization of RXR on mitochondria was also demonstrated by immunoblotting analysis showing that RXR was accumulated in the mitochondria-enriched heavy membrane fraction when cells were treated with TPA or MM1453. Pretreatment of cells with 9-*cis* RA inhibited the accumulation of RXR (Figure 1C). The mitochondrial localization of RXR is depended on TR3 expression as RXR failed to reside on mitochondria in cells expressing TR3 antisense RNA (Figure 1C), suggesting that RXR may target mitochondria as TR3/RXR heterodimer. 9-*cis* RA also prevented TPA-induced TR3 cytoplasmic localization and cytochrome c release in LNCaP cells (Figure 1D).

Pretreatment of LNCaP cells with 9-*cis* RA or MM11237 also prevented release of cytochrome c from mitochondria and apoptosis induced by TPA and MM1453 in LNCaP cells (Figure 2).

Together, our studies demonstrate that TR3 translocates from the nucleus to the cytoplasm as TR3/RXR heterodimer and that a nuclear export sequence in the RXR is required for the process.

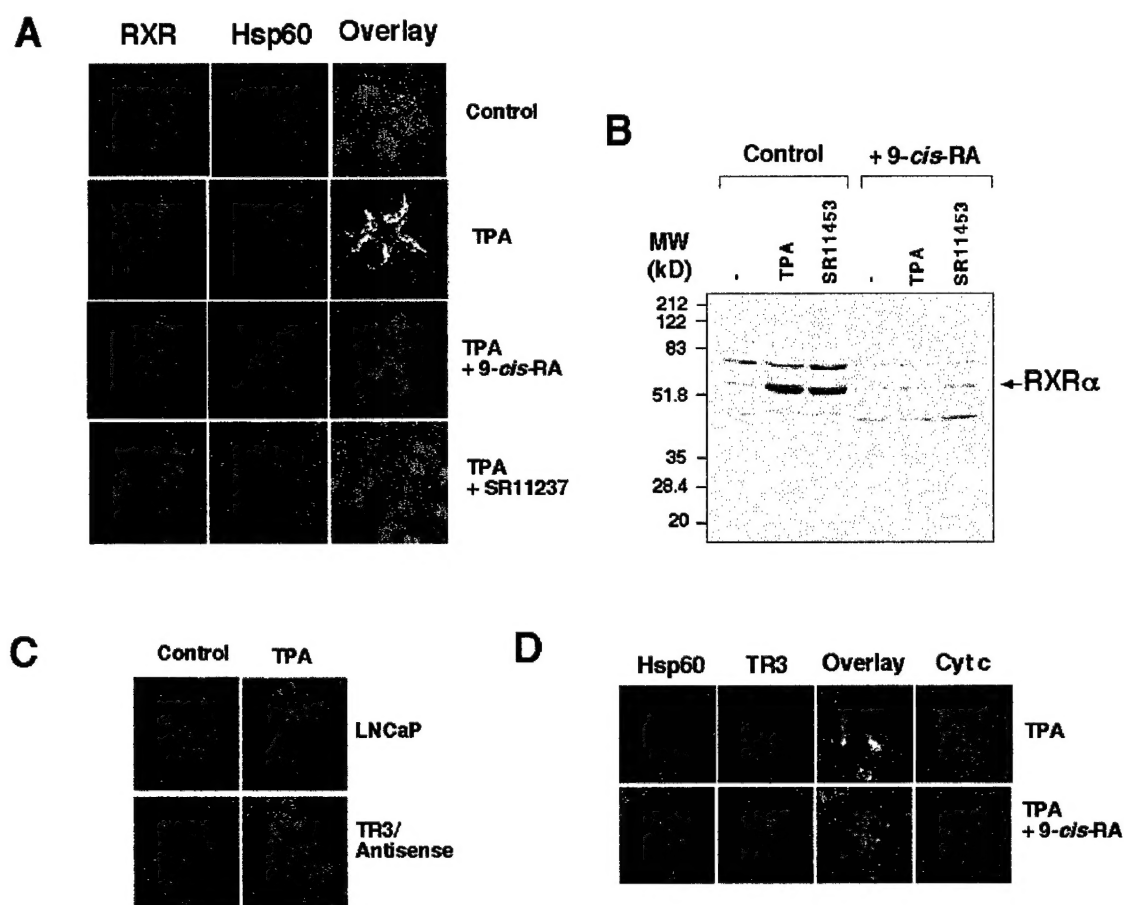


Figure 1. Localization of RXR in mitochondria in a TR3-dependent manner. A. Regulation of RXR mitochondrial localization by apoptosis inducer TPA and RXR ligands. LNCaP cells were pre-

treated with or without RXR ligands 9-*cis* RA (10^{-7} M) or SR11237 (10^{-6} M) for 12 hr before TPA treatment (1 hr), then immuno-stained with anti-RXR antibody followed by Cy3-conjugated secondary antibody (Sigma) to detect RXR, or with anti-Hsp60 followed by Cy5-conjugated secondary antibody (Sigma) to detect mitochondria. RXR and mitochondria (Hsp60) were visualized using confocal microscopy and the two images were overlaid (overlay). **B.** Apoptotic stimuli induce accumulation of RXR in mitochondria. LNCaP cells were treated with TPA (100 ng/ml) or MM11453 (10^{-6} M) for 3 hr in the absence or presence of 9-*cis* RA (10^{-6} M), and the HM fraction was analyzed for expression of RXR by Western blotting. **C.** Mitochondrial localization of RXR is TR3 dependent. LNCaP cells or LNCaP cells stably expressing TR3 antisense RNA (TR3/Antisense) were treated with or without TPA for 1 hr, then immuno-stained with anti-RXR antibody followed by Cy3-conjugated secondary antibody (Sigma) to detect RXR. **D.** 9-*cis* RA inhibits TPA-induced TR3 mitochondrial localization and cytochrome c release in LNCaP cells.

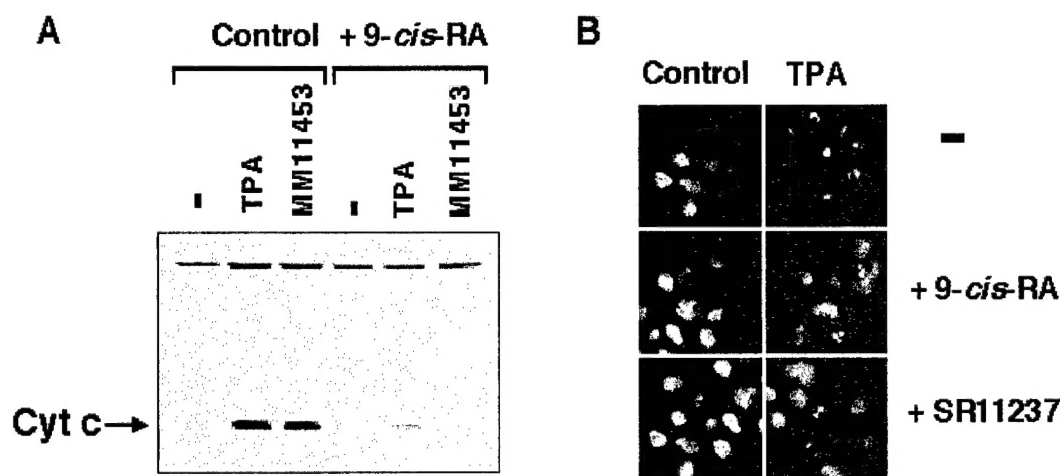


Figure 2. Inhibition of cytochrome c release and apoptosis by RXR agonists. A. Inhibition of cytochrome c release. LNCaP cells were treated with TPA (100 ng/ml) or MM11453 (10^{-6} M) in the presence or absence of 9-*cis* RA (10^{-7} M) for 3 hours. Cytosolic fractions were then prepared and analyzed for expression of cytochrome c (Cyt c) by Western blotting. A nonspecific band at ~ 70 kd served as a control for equal loading of proteins. **B. Inhibition of apoptosis.** LNCaP cells were treated with MM002 (10^{-6} M) in the presence or absence of 9-*cis* RA (10^{-6} M) or SR11237 (10^{-6} M) for 36 hours and nuclei were stained by DAPI.

2. Interaction between TR3 and Bcl-2

Members of the Bcl-2 family are important regulators of cell death and survival (6,7). Many of which, such as Bcl-2, are located predominantly in the outer mitochondrial membrane (6,7). We investigated the possibility that TR3 targeted mitochondria by interacting with Bcl-2 that is known to reside on the outer membrane of mitochondria. TR3/ Δ DBD, which constitutively resides on mitochondria (3), was analyzed in a co-immunoprecipitation (Co-IP) assay for its interaction with Bcl-2 by transfecting it into human embryonic kidney cell line 293T alone or with Bcl-2 expression vector. Co-IP assay showed that a significant amount of TR3/ Δ DBD was co-precipitated with Bcl-2 by anti-Bcl-2 antibody (Figure 3a). In a reporter gene assay in CV-1 cells, transactivation of TR3 on its responsive element (NurRE-tk-CAT) was

its ability of binding Bcl-2, failed to enhance DC1 binding to Bcl-2. Thus, TR3 interacts with Bcl-2 in a manner that is different from other Bcl-2 family proteins.

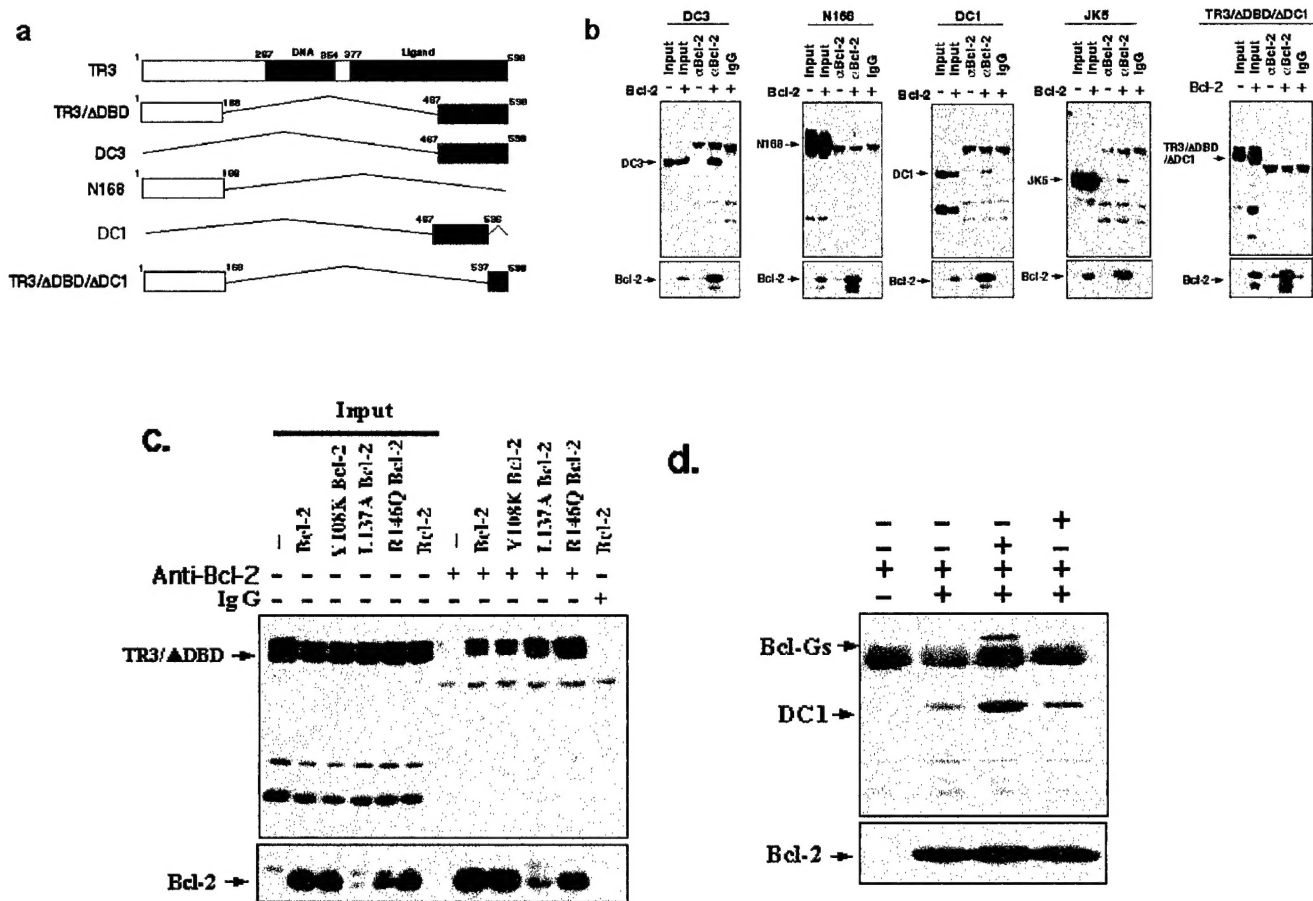


Figure 4. Mutational analysis of TR3 and Bcl-2. **a.** Schematic representation of TR3 mutants. DNA-binding and ligand-binding domains of TR3 are indicated. **b.** *In vivo* Co-IP. The indicated TR3 mutants were analyzed for their interaction with Bcl-2 by Co-IP assay as described in Fig. 3a. **c.** The hydrophobic groove of Bcl-2 is not required for its binding to TR3/ΔDBD. Bcl-2 mutant, Y108 KBcl-2, L137A Bcl-2 or R146QBcl-2, was analyzed for their interaction with GFP-TR3/ΔDBD in 293T cells by Co-IP as described in Fig. 3a. **d.** Competition assay. Bcl-2 was co-transfected into 293T cells with either GFP-DC1 or GFP-Bcl-Gs or a Bcl-Gs mutant and the Co-IP was performed as described in Fig. 3a.

Bcl-2 mediates TR3 mitochondrial targeting and apoptosis. We studied whether TR3/ΔDBD targets mitochondria via its interaction with Bcl-2. TR3/ΔDBD expressed in 293T cells exhibited a diffused distribution pattern (Figure 5a), but colocalized with Bcl-2 and heat shock protein 60 (Hsp60), a mitochondrial specific protein when Bcl-2 and TR3/ΔDBD were coexpressed. (Figure 5a). Immunoblotting of the mitochondria-enriched heavy membrane (HM) fractions showed a significantly enhanced accumulation of TR3/ΔDBD in mitochondria when Bcl-2 was coexpressed (Figure 5b). These results demonstrate that Bcl-2 acts as a receptor for TR3 to target mitochondria. We next studied the involvement of TR3/Bcl-2 interaction in TR3-induced cyto c release. In the absence of Bcl-2 cotransfection, TR3/ΔDBD did not release cyto c from mitochondria as determined by confocal microscopy analysis (Figure 5c). However, on

Bcl-2 cotransfection TR3/ Δ DBD and Bcl-2 colocalized and cyto c was released (Figure 5c). DAPI staining indicated that TR3/ Δ DBD only caused nuclear fragmentation or condensation when Bcl-2 was cotransfected (Figure 5d). Thus, the TR3/ Δ DBD-Bcl-2 interaction is required to induce cyto c release and apoptosis.

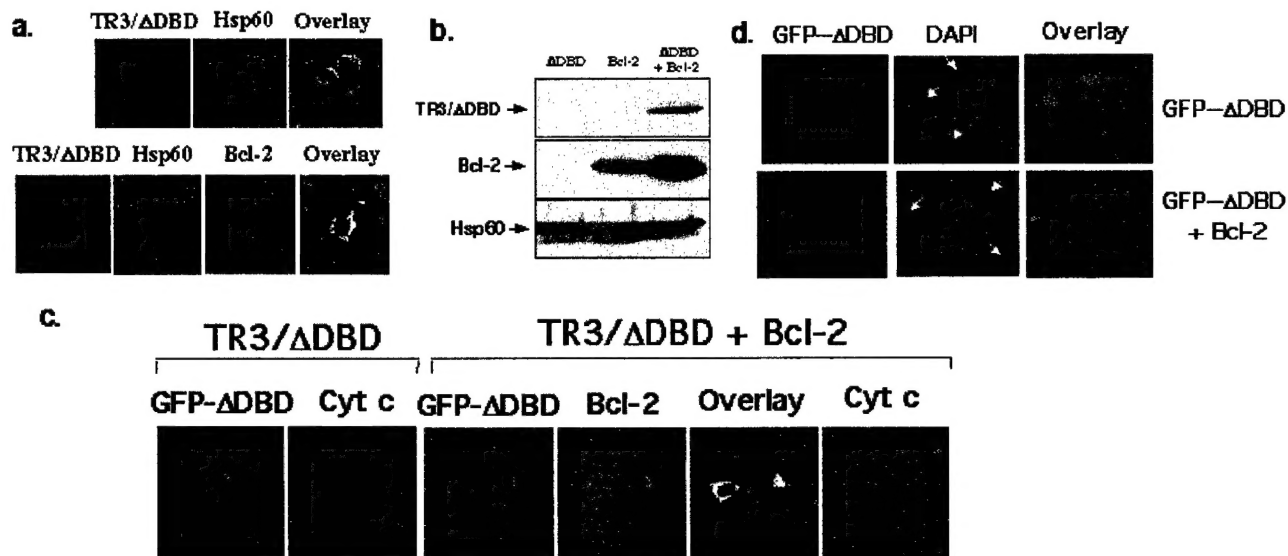


Figure 5. Bcl-2 expression promotes TR3/ Δ DBD mitochondrial localization and its apoptotic effect. **a.** Confocal microscopy analysis. GFP-TR3/ Δ DBD and Bcl-2 were transfected into 293T cells alone or together. Cells were immunostained with anti-Bcl-2 antibody followed by Cy3-conjugated secondary antibody, or with anti-Hsp60 antibody followed by Cy5-conjugated secondary antibody. Bcl-2, TR3/ Δ DBD and mitochondria (Hsp60) were visualized using confocal microscopy. **b.** Western blotting. The heavy membrane fractions were prepared and analyzed for accumulation of TR3/ Δ DBD by Western blotting using anti-GFP antibody. **c.** Bcl-2 is required for TR3 to induce cyto c release. GFP-TR3/ Δ DBD and Bcl-2 were transfected into 293T cells alone or together. Cells were immunostained with anti-cyto c (cyt c) antibody followed by Cy5-conjugated secondary antibody, or with anti-Hsp60 followed by Cy3-conjugated secondary antibody. Cyto c, TR3/ Δ DBD, and mitochondria (Hsp60) were visualized using confocal microscopy. **d.** Bcl-2 is required for TR3/ Δ DBD to induce apoptosis. Nuclei of 293T cells transfected with GFP-TR3/ Δ DBD alone or together with Bcl-2 were stained by DAPI. GFP- Δ DBD expression and nuclear morphology were visualized by fluorescence microscopy. Arrows indicate cells displaying nuclear condensation and fragmentation.

REPORTABLE OUTCOMES

1. Dawson, M.I., Hobbs, P., Peterson, V., Leid, M., Lange, C., Feng, K., Chen, G., Gu, J., Li, H., Kolluri, S., Zhang, X-k., Zhang, Y., and Fontana, J. Induction of apoptosis in cancer cells by a novel analog of 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN) lacking retinoid receptor transcriptional activation activity. *Cancer Research*. 61:4723-4730, 2001.
2. Zhang, X-k., Vitamin A and apoptosis in prostate cancer. *Endo. Related Cancer*. In Press. 2002.
3. Kolluri, S., Lin, B., Cao, X., Li, H., Lin, F., James, S., and Zhang, X-k. Nuclear orphan receptor TR3 induces apoptosis and proliferation by distinct pathways. *The Keystone Symposium on Nuclear Receptor Superfamily*. Snowbird, Utah, April 13-29, 2002

4. Zhang, X-k., Kolluri, S., Cao, X., Li, H., Lin, B., and Dawson, M.I. Subcellular Localization of Orphan Receptor TR3 Defines its Biological Activities. The FASEB Summer Conference on Retinoids. Tucson, Arizona, June 22-27, 2002.

CONCLUSIONS

In summary, we have conducted enormous amount of experiments to study the regulation of TR3 nuclear export, mitochondrial targeting and apoptosis induction in prostate cancer cells. Our results demonstrate that the migration of TR3 from the nucleus to the cytoplasm requires RXR through their heterodimerization. In addition, we show that the TR3 cytoplasmic localization and apoptotic effect are regulated by RXR ligands. These data not only enhance our understanding the molecular mechanism of TR3 nuclear export but also provide important information for developing RXR ligands for regulating apoptosis of prostate cancer cells. In addition, we show that TR3 interacts with Bcl-2 and that the interaction between TR3 and Bcl-2 is essential for TR3 to target mitochondria and to induce apoptosis. Our observation that the TR3-Bcl-2 interaction converts Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule provides novel molecular basis for inducing apoptosis of Bcl-2-expressing cancer cells. The overall plan for the next year of research remains the same as proposed in the original grant application.

REFERENCES

1. Liang, J.Y., Fontana, J.A., Rao, J.N., Ordonez, J.V., Dawson, M.I., Shroot, B., Wilber, J.F. And Feng, P. Synthetic retinoid CD437 induces S-phase arrest and apoptosis in human prostate cancer cells LNCaP and PC-3. Prostate. 1999. **38**: 228-36.
2. Sun, S. Y., Yue, P., and Lotan, R., Implication of multiple mechanisms in apoptosis induced by the synthetic retinoid CD437 in human prostate carcinoma cells, *Oncogene*, **19**, 4513-22. (2000).
3. Li, H., Kolluri, S. K., Gu, J., Dawson, M. I., Cao, X., Hobbs, P. D., Lin, B., Chen, G., Lu, J., Lin, F., Xie, Z., Fontana, J. A., Reed, J. C., and Zhang, X., Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3 [see comments] [comment], *Science*, **289**, 1159-64 (2000).
4. Forman, B. M., Umesono, K., Chen, J., and Evans, R. M., Unique response pathways are established by allosteric interactions among nuclear hormone receptors, *Cell*, **81**, 541-50 (1995).
5. Perlmann, T., and Jansson, L., A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1, *Genes Dev*, **9**, 769-82 (1995).
6. Adams, J. M., and Cory, S., The Bcl-2 protein family: arbiters of cell survival, *Science*, **281**, 1322-6 (1998).
7. Reed, J. C., Bcl-2 family proteins, *Oncogene*, **17**, 3225-36 (1998).
8. Guo, B., Godzik, A., and Reed, J. C., Bcl-G, a novel pro-apoptotic member of the Bcl-2 family, *J Biol Chem*, **276**, 2780-5. (2001).

APPENDICES

1. Dawson, M.I., Hobbs, P., Peterson, V., Leid, M., Lange, C., Feng, K., Chen, G., Gu, J., Li, H., Kolluri, S., Zhang, X-k., Zhang, Y., and Fontana, J. Apoptosis induction in cancer cells by a novel analog of 6-[3- (1-Adamantyl) -4- hydroxyphenyl} -2- naphthalenecarboxylic acid (AHPN) lacking retinoid receptor transcriptional activation activity. *Cancer Research*. 61:4723-4730, 2001.
2. Zhang, X-k., Vitamin A and apoptosis in prostate cancer. *Endo. Related Cancer*. In Press. 2002.

Apoptosis Induction in Cancer Cells by a Novel Analogue of 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic Acid Lacking Retinoid Receptor Transcriptional Activation Activity¹

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ABSTRACT

The retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN) is reported to have anticancer activity *in vivo*. Induction of cell cycle arrest and apoptosis in cancer cell lines refractory to standard retinoids suggests a retinoid-independent mechanism of action for AHPN. Conformational studies suggested that binding of AHPN does not induce an unusual conformation in retinoic acid receptor (RAR) γ . The 3-chloro AHPN analogue MM11453 inhibited the growth of both retinoid-resistant (HL-60R leukemia, MDA-MB-231 breast, and H292 lung) and retinoid-sensitive (MCF-7 breast, LNCaP prostate, and H460 lung) cancer cell lines by inducing apoptosis at similar concentrations. Before apoptosis, MM11453 induced transcription factor TR3 expression and loss of mitochondrial membrane potential characteristic of apoptosis. MM11453 lacked the ability to significantly activate RARs and retinoid X receptor α to initiate (TREp)₂-tk-CAT reporter transcription. These results, differential proteolysis-sensitivity assays, and glutathione S-transferase-pulldown experiments demonstrate that, unlike AHPN or the natural or standard synthetic retinoids, MM11453 does not behave as a RAR or retinoid X receptor α transcriptional agonist. These studies strongly suggest that AHPN exerts its cell cycle arrest and apoptotic activity by a signaling pathway independent of retinoid receptor activation.

INTRODUCTION

The natural RAs³ and their synthetic analogues are being investigated as chemotherapeutic agents because they inhibit proliferation, induce apoptosis in cancer cells, and retard tumor xenograft growth (1). These standard retinoids exert their antiproliferative effects by influencing the transcriptional activity of RAR and RXR subtypes α , β , and γ (reviewed in Ref. 2). Retinoids complexed to a RXR/RAR can activate or repress gene transcription from RA response elements in the promoter of retinoid-sensitive genes. A retinoid bound to an RXR can modulate activation by other transcription factors with which it dimerizes (2). Retinoid receptor-ligand complexes also compete with other transcription factors for coactivator proteins (3, 4), whereas nonliganded dimers compete for corepressors (5).

The diversity from the six subtypes and variations in their expres-

sion patterns (2, 6-9), response element sequences, intermediary proteins, and other transcription factors (2) led to the identification of receptor-selective retinoids to enhance efficacy by reducing the systemic toxicity associated with retinoids activating all receptors (10). Receptor class and subtype-selective compounds (reviewed in Refs. 1 and 11) also provide a means for studying individual receptor-signaling pathways.

On evaluating RAR γ -selective retinoids, we observed that AHPN (CD437 [1] in Fig. 1; Ref. 12) rapidly caused detachment of retinoid-sensitive MCF-7 breast and NIH:OVCAR-3 ovarian cancer cells (13, 14). This atypical retinoid activity extended to retinoid-resistant lines, including MDA-MB-231 breast cancer and HL-60R leukemia (13). AHPN induced cyclin-dependent kinase inhibitor p21^{WAF1/CIP1} expression (13), G₀-G₁ cell cycle arrest (13), and apoptotic events, such as caspase activation, *gadd45* expression (15), poly(adenosyl diphosphate-ribose) polymerase cleavage, and DNA fragmentation (13). Interestingly, apoptosis occurred in the absence of functional tumor suppressor *p53* (13), the gene for which is mutated in many cancers (16). Apoptosis by AHPN and its derivatives and analogues was subsequently observed in other lines derived from tumors and their metastases (17-25).

The efficacy against retinoid-resistant cancer cells prompted studies on how AHPN induces apoptosis. To reduce complications, we conducted mechanistic studies in cells lacking functional retinoid receptors (26) and used apoptotic AHPN analogues lacking retinoid agonist transactivation activity, such as MM11453 [2]. MM11453 induced apoptosis by a cascade that included mitochondrial translocation of transcription factor TR3/nur77/NGFIB-II (TR3), cytochrome *c* release, caspase activation, and DNA fragmentation (27). Binding of MM11453 to RARs and RXR α did not cause the conformational changes of AHPN that led to corepressor loss and coactivator recruitment. We report here the characterization and anticancer activity of MM11453, the prototype for new nonretinoidal apoptotic agents with potential for cancer treatment.

MATERIALS AND METHODS

Retinoids. AHPN [1] was prepared by modifying a reported procedure (28). AHPN (MM11453) [2] was synthesized as follows. The biaryl bond was introduced by palladium(0)-catalyzed coupling between 3-(1-adamantyl)-4-benzyloxybenzeneboronic acid and ethyl 6-bromo-3-chloro-2-naphthalenecarboxylate [palladium(triphenylphosphine)₄ (Aldrich, St. Louis, MO), aqNa₂CO₃, dimethoxyethane, reflux, 6 h], followed by chromatography (6% EtOAc/hexane on silica gel) to give the benzyl-protected ethyl ester of MM11453 (66%). Benzyl group cleavage [BBR₃, CH₂Cl₂, -78°C, 2 h] to the phenol (91%) and ester group hydrolysis (aq NaOH, ethanol, 90°C, 2 h; aq HCl) gave MM11453 (95%) as a white powder, melting point 294°C-296°C (decomp.). IR (KBr): 3200, 1706, 1277, 1244, 991, 815, and 680 cm⁻¹. ¹H nuclear magnetic resonance (300 MHz, Me₂SO-*d*₆, δ): 1.75, 2.06, 2.17 (s, 6, adamantyl CH₂; s, 3, adamantyl CH;

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³ The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid; aq, aqueous; GST, glutathione S-transferase; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; DPSS, differential protease sensitivity assay; TRE, thyroid hormone receptor response element; TREp, palindromic TRE; PF, protease-resistant fragment; Hsp, heat shock protein; LBD, ligand-binding domain; Met, methionine; NCoR, nuclear receptor corepressor; Rh123, rhodamine green; TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid.

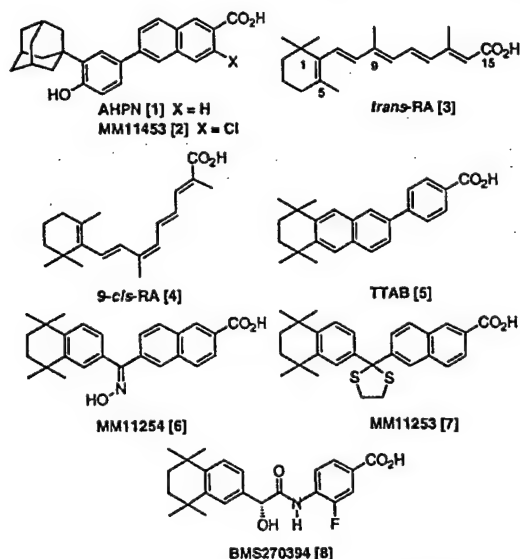


Fig. 1. AHPN [1], MM11453 [2], *trans*-RA [3], 9-*cis*-RA [4], TTAB [5], MM11254 [6], MM11253 [7], and BMS270394 [8].

s, 6, adamantyl CH₂), 6.97 (d, $J = 9.0$ Hz, 1, ArH-5), 7.51 (s, 1, ArH-2), 7.52 (d, $J = 9.0$ Hz, 1, ArH-6), 7.98 (d, $J = 9.0$ Hz, 1, NapH-8), 8.06 (s, 1, NapH-5), 8.25 (d, $J = 8.6$ Hz, 1, NapH-7), 8.27 (s, 1, NapH-4), 8.60 (s, 1, NapH-1), 9.68 (s, 1, ArOH). High-resolution mass spectrometry for C₂₇H₂₅ClO₃ (M⁺): calculated, 432.1492; found, 432.1492. *trans*-RA [3] was purchased (Sigma Chemical Co.), as was [11,12-³H]₂9-*cis*-RA (specific activity, 43 Ci/mmol; DuPont NEN, Boston, MA). 9-*cis*-RA [4] was prepared as reported (29).

Computational Analysis. CAChe Software (Fujitsu, Beaverton, OR) was used to identify low-energy conformers within 2 kcal of the global energy minimum (MM3 force field, conjugate-gradient minimization, 30° search label variation, exclusion of ≥ 9 Å van der Waals interactions, and energy change < 0.001 kcal/mol). Conformers were superimposed by using least-squares rigid fit of atoms corresponding to the 1, 5–9, and 15 carbon molecules of *trans*-RA.

Receptor Transcriptional Activation. CV-1 cells (1,000 per well) were grown in DMEM (Irving Scientific, Santa Ana, CA) with 10% charcoal-treated FCS (Tissue Culture Biologicals, Tulare, CA) for 16–24 h before transfection, as described (30, 31). Briefly, 100 ng of (TREp)₂-*tk*-CAT reporter, β -galactosidase expression vector pCH 110 (Pharmacia, Piscataway, NJ), and a RAR expression vector (or 20 ng of RXR α) were mixed with carrier DNA (pBlue-script; Stratagene, La Jolla, CA) to give 1 μ g of total DNA/well. CAT activity was normalized using β -galactosidase as the control. Activation after subtraction of constitutive activity is expressed relative to that of 1.0 μ M *trans*-RA for RARs (100%) or 1.0 μ M 9-*cis*-RA for RXR α (100%) and represents the average of three determinations.

Receptor Binding. Competitive radioligand binding on crude bacterial lysates at 0°C for 2 h used ~ 25 μ mol of recombinant human RAR subtype or mouse RXR α -GST fusion proteins in 200 μ l of binding buffer [10 mM HEPES (Sigma Chemical Co.; pH 7.8), 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, and 10% glycerol] with 1–2 nM [³H]9-*cis*-RA (43 Ci/mmol). Bound [³H]9-*cis*-RA was isolated (Sephadex G-50; Pharmacia) and counted. Nonspecific [³H]9-*cis*-RA binding at 1 μ M nonlabeled 9-*cis*-RA generally was $< 10\%$ of total label bound.

DPSA. [³⁵S]Met-labeled RAR α , RAR β , RAR γ , and RXR α , prepared by *in vitro* transcription/translation (32), were used in DPSA as described (33). [³⁵S]Met-labeled receptors were incubated with 0.1% ethanol alone, 1.0 μ M 9-*cis*-RA, or MM11453 for 30 min at 0°C. Limited proteolysis (trypsin-tosyl phenylalanyl chloromethyl ketone; Sigma Chemical Co.) for 15 min at 22°C, followed by termination by Laemmli sample buffer and boiling and separation

(10% acrylamide gel under denaturing conditions), afforded PFs for visualization by autoradiography (33, 34).

GST-Pulldown. Experiments were performed as described using GST-p300 1–450 (35) and GST-NCoR 2110–2453 (36) fusion proteins and [³⁵S]Met-labeled human RAR γ .

Cell Lines and Culture. RA-resistant HL-60R cells, having a mutant RAR α that does not significantly bind *trans*-RA and lacking RAR β and RAR γ (26), and MDA-MB-231 cells were grown as described (20). MCF-7, LNCaP prostate, H460 and retinoid-resistant H292 lung cancer cells and Jurkat lymphoma cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Irving Scientific) with 10% charcoal-treated FCS.

Cell Growth Inhibition. HL-60R and MDA-MB-231 cells (50,000 and 100,000 per well, respectively) and 0.1–1.0 μ M MM11453, AHPN, or Me₂SO alone were incubated for 24 or 120 h (72-h medium change), respectively. Results are expressed relative to Me₂SO control as mean \pm SE of triplicate experiments. SEs were $< 10\%$. MCF-7, LNCaP, H292, and H460 cells (3,000 per well in 96-well plates) were treated with 1.0 μ M MM11453, AHPN, *trans*-RA, or ethanol alone for 48 h before viable cell numbers were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (7, 9, 27). Data shown are representative of three experiments.

Apoptosis Detection. DNA fragmentation and apoptotic bodies were assessed in at least 500 HL-60R or MDA-MB-231 cells after incubation with MM11453 for 24 or 120 h, respectively, as described above, and acridine orange staining (15). The percentage of apoptotic cells was expressed relative to the Me₂SO control as the mean \pm SE of triplicate experiments. MCF-7, LNCaP, H292, H460, and Jurkat cells (3,000 per well) were treated with 1.0 μ M MM11453, *trans*-RA, or ethanol alone for 48 h, trypsinized, washed (PBS), fixed (3.7% paraformaldehyde), and stained with 4',6-diamidino-2-phenylindole (1 μ g/ml) to visualize nuclei by fluorescent microscopy (21). Cells with apoptotic nuclear morphology were scored in each 400-cell sample using a fluorescence microscope. The data are representative of three experiments.

Northern Analysis. Total RNAs were prepared (RNeasy Mini kit; Qiagen, Germany), and TR3 expression was determined on 30 μ g of total RNA from each line treated with 1.0 μ M MM11453, *trans*-RA, or ethanol alone. Blotting conditions were as described (27) with β -actin expression as the control.

TR3 Mitochondrial Targeting. The expression vector for TR3/ Δ DBD-GFP, a TR3 mutant lacking the DNA-binding domain fused to the green fluorescent protein expression vector, was transiently transfected into H460 cells, as described for LNCaP cells (27). Cells were treated with 1.0 μ M MM11453 or ethanol alone for 6 h and then immunostained with anti-Hsp60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Cy3-conjugated secondary antibody (Sigma Chemical Co.) to indicate mitochondria to which Hsp60 is restricted. Confocal microscopy was used to detect TR3/ Δ DBD-GFP (green fluorescence) and Hsp60 (red). Images were overlaid to show colocalization.

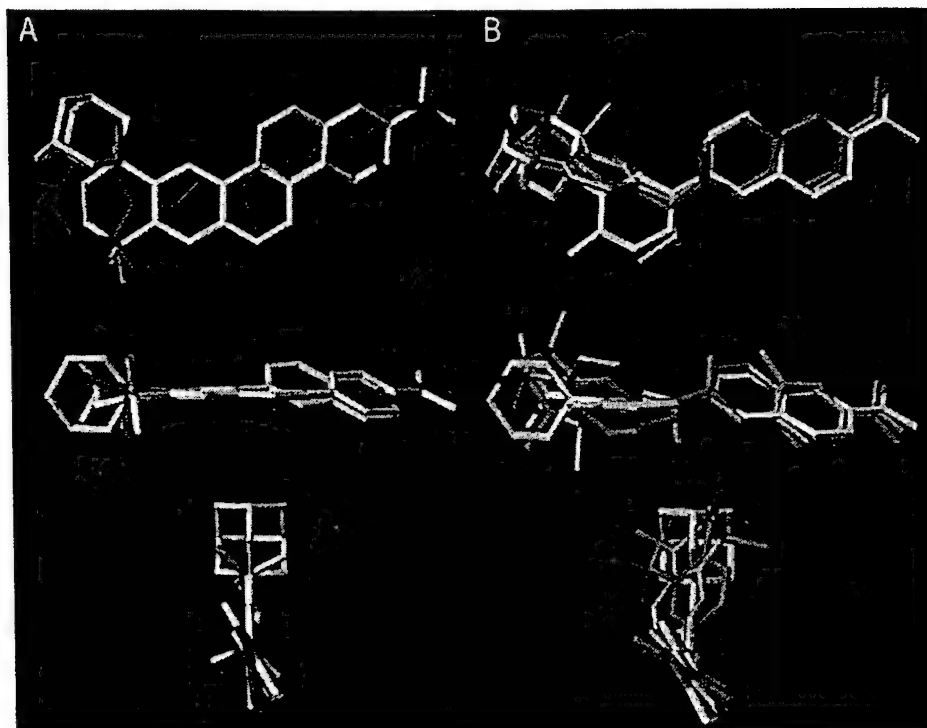
Mitochondrial Membrane Potential Assay. LNCaP, MCF-7, and MDA-MB-231 cells (10,000,000) were treated with 1.0 μ M MM11453 for 18 h before incubation with 5 μ g/ml Rh123 for 30 min at 37°C. Rh123-fluorescing cells were scored depolarized by flow cytometry (FACScalibur system; BD Biosciences, San Jose, CA; Ref. 37). The data shown are representative of three experiments. Wild-type Jurkat cells or Jurkat cells stably expressing either Bcl-2 or control vector (38) were treated similarly.

RESULTS

Close-Fitting of Energy-minimized AHPN and Retinoid Conformers. Energy-minimized conformers of AHPN [1], RAR-selective *trans*-RA [3], and RAR-selective TTAB [5] (39) were overlapped. The *trans*-RA conformer was that reported in the RAR γ LBD (40, 41). Three orthogonal views of these overlaps are shown in Fig. 2A. The major structural difference was the 1-adamantyl group of AHPN, which extended 2.2 Å more than the *trans*-RA 18-methyl group. In Fig. 2B, the energy-minimized conformers of RAR γ -selective agonists AHPN and MM11254 [6], a (Z)-oxime (14) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl-carbonyl)-2-naphthalenecarboxylic acid (42), are shown overlapped with RAR γ -selective agonist BMS270394 [8], as found in the ligand-binding pocket of crystallized holo-RAR γ (40). The

⁴ Assay conducted at The Burnham Institute, La Jolla, CA, under a license agreement with Ligand Pharmaceuticals, San Diego, CA.

Fig. 2. Comparison of energy-minimized AHPN and retinoid conformers. Conformational analysis was performed as described in "Materials and Methods." A, orthogonal views of superimposed conformers of AHPN (blue), *trans*-RA (red), and TTAB (yellow). B, superimposed conformers of AHPN (blue), MM11254 (green), and BMS270394 (magenta).



AHPN 1-adamantyl group overlaps the saturated portion of the 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene rings of both RAR γ -selective retinoids, and the AHPN phenolic oxygen is near the oxygen molecules in the oxime group of MM11254 (2.5 Å) and the bridge hydroxyl of BSM270394 (3.9 Å). Such hydroxyl groups are reported to confer RAR γ selectivity by hydrogen bonding to the Met-272 sulfur molecule of RAR γ (41). Placement of these overlapped conformers (Fig. 2B) in the RAR γ ligand-binding site gives ligand O–Met-272–S distances of 4.09, 2.55, and 3.32 Å,

respectively. These studies suggest that binding of AHPN to RAR γ occurs in the same manner as that of standard retinoid agonists.

MM11453 Lacked RAR Transcriptional Activation of AHPN. Although originally reported as RAR γ selective on the (TREpal) $_2$ -tk-CAT reporter in cotransfected HeLa cells (12), we observed on the (TREpal) $_2$ -tk-CAT in CV-1 cells (4) that high RAR γ selectivity occurred at 0.1 μ M and below (14). At 0.5–1.0 μ M, at which the natural retinoid *trans*-RA [3] inhibits retinoid-sensitive cancer cells, AHPN significantly activated RAR β . At 1.0 μ M AHPN, reporter activation by RAR α , RAR β , and RAR γ was 9, 37, and 54%, respectively, of that caused by 1.0 μ M *trans*-RA (Fig. 3). Unlike 1.0 μ M *trans*-RA or 9-*cis*-RA, 1.0 μ M MM11453 did not adequately activate any RAR subtype or RXR α to induce even modest (TREpal) $_2$ -tk-CAT transcription. MM11453 did not activate RAR α or RXR α and only activated RAR β and RAR γ to 5 and 10%, respectively, of that of *trans*-RA or 13 and 19%, respectively, of that of AHPN. Thus, MM11453 is an analogue with substantially reduced capacity for RAR activation.

Retinoid Receptors Bound MM11453. Competitive ligand binding was used to determine whether MM11453 bound directly to RARs and RXR α . MM11453 at 1.0 μ M displaced $61 \pm 6\%$ of [3 H]*9-cis*-RA bound to RAR γ , whereas displacement from other receptors was lower [RAR α ($11 \pm 2\%$), RAR β ($25 \pm 5\%$), and RXR α ($18 \pm 5\%$); Fig. 4].

MM11453 Did Not Induce an Agonist-bound RAR Conformation. DPSA on 9-*cis*-RA-bound RAR α , RAR β , and RAR γ produced 27-kDa PF27 α , 35-kDa PF35 β , and 32-kDa PF32 γ , respectively (Lane 3 in Fig. 5, A–C). DPSA on AHPN-bound RARs produced the same fragments (data not shown). These PFs were not observed on incubation with ethanol or MM11453 (Lanes 2 and 4, respectively, in Fig. 5, A–C). Unlike 9-*cis*-RA, neither MM11453 nor AHPN altered the proteolytic sensitivity of RXR α (data not shown). The lack of PFs from RAR-MM11453 complexes suggests that MM11453 does not

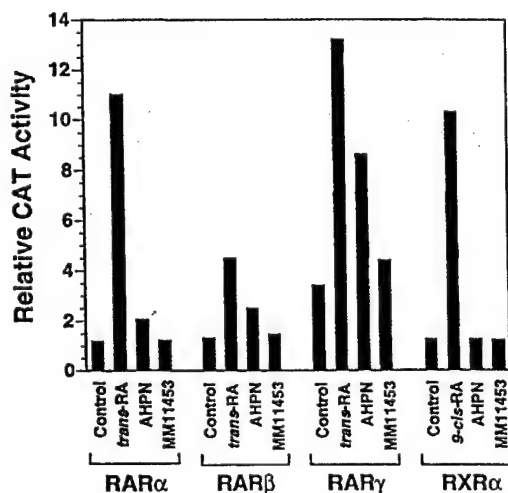


Fig. 3. Transcriptional activation of retinoid receptors by MM11453 on the (TREpal) $_2$ -tk-CAT reporter. CV-1 cells were transiently transfected as described in "Materials and Methods," treated with 1.0 μ M MM11453, AHPN, *trans*-RA, or 9-*cis*-RA, and assayed for CAT activity after 24 h. Reporter gene activation is expressed relative to 1.0 μ M *trans*-RA on the RARs or 1.0 μ M 9-*cis*-RA on RXR α .

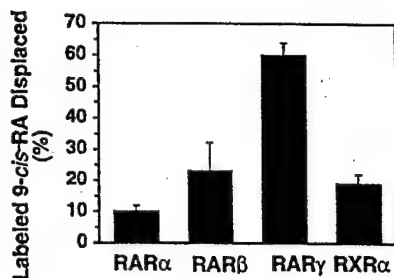


Fig. 4. Binding affinity of MM11453 to recombinant RAR and RXRα. Competition radioligand binding was conducted as described in "Materials and Methods." The data represent the means ($n = 3$) of the percentages of [$^{11,12-3}H$]9-*cis*-RA bound that were inhibited by 1.0 μM MM11453; bars, SE.

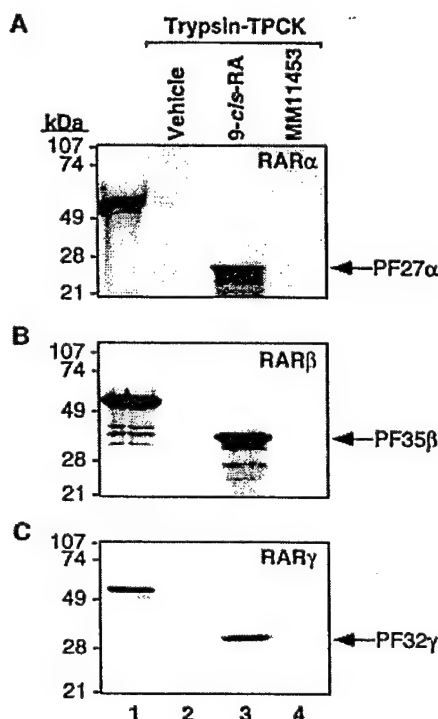


Fig. 5. MM11453 is not a RAR agonist. A, DPSA on [^{35}S]Met-labeled RARα in ethanol or 1 μM 9-*cis*-RA or MM11453. The migration of the 9-*cis*-RA-induced PF27α of RARα is indicated. In B and C, DPSA on RARβ and RARγ, respectively, were conducted as in A and "Materials and Methods." Positions of RARβ PF35β and RARγ PF32γ are indicated. Left, marker migration (molecular mass).

promote an agonist-bound conformation. Similar to RARγ-selective antagonist MM11253,⁵ MM11453 did not prevent RAR/RXR agonist 9-*cis*-RA from inducing this conformation in RARα, RARβ, or RARγ (data not shown).

MM11453 Failed to Dissociate Corepressor NCoR-RARγ in Vitro. GST-pull-down was used to test whether MM11453 dissociated NCoR (5) from RARγ, as 9-*cis*-RA does. As indicated (Fig. 6A), 9-*cis*-RA (Lane 3), but not MM11453 (Lane 4) or vehicle (Lane 2), disrupted the NCoR-RARγ complex.

MM11453 Failed to Recruit Coactivator p300 to RARγ. We compared the abilities of MM11453 and 9-*cis*-RA (36) to recruit p300

(43) to RARγ. Vehicle (Lane 2 in Fig. 6B) or MM11453 (Lane 4) did not enhance p300 recruitment, whereas 9-*cis*-RA did (Lane 3). These findings, which agree with results on MM11453 in RARγ DPSAs (Fig. 5) and corepressor-dissociation experiments (Fig. 6A), confirm that MM11453 does not induce a RARγ-agonist conformation.

MM11453 Inhibited Cancer Cell Growth. Increasing evidence, including retinoid-resistant cancer cell growth inhibition (13, 27), suggests that AHPN action is independent of retinoid receptors (21, 44). Cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were conducted to show that MM11453 inhibited growth similarly. MM11453 inhibited HL-60R and MDA-MB-231 growth with IC_{50} s of 0.17 and 0.32 μM (Fig. 7, A and B), respectively, compared with AHPN values of 0.15 and 0.30 μM , respectively. Inhibition by 1.0 μM *trans*-RA was $\leq 5\%$ (13). The effects of MM11453 on H460, H292, LNCaP, and Jurkat cells were then examined. As shown (Fig. 7C), 1.0 μM MM11453 significantly reduced growth by 70, 46, 64, and 70%, respectively, whereas 1.0 μM *trans*-RA reduced H460 growth by 15% and had no evident effect on the other lines (0–3%). Some of us reported previously that 1.0 μM AHPN for 48 h inhibited the growth of H460, H292, and LNCaP cells by $62 \pm 6\%$ (21), $53 \pm 5\%$ (21), and $100 \pm 5\%$ (25), respectively, whereas Jurkat growth was inhibited by 84% and $80 \pm 3\%$ after 24 and 96 h, respectively (22). Thus, both AHPN and MM11453 similarly retard the growth of these cell lines.

MM11453 Induced Cancer Cell Apoptosis. The MM11453 EC_{50} s for inducing nuclear fragmentation in HL-60R and MDA-MB-231 cells were 0.12 and 0.13 μM , respectively (Fig. 7, D and E), which are similar to AHPN EC_{50} s of 0.07 and 0.35 μM , respectively (13). HL-60R apoptosis inhibition by 1.0 μM MM11453 and AHPN was $82 \pm 3\%$ and $91 \pm 4\%$, respectively, and MDA-MB-231 apoptosis

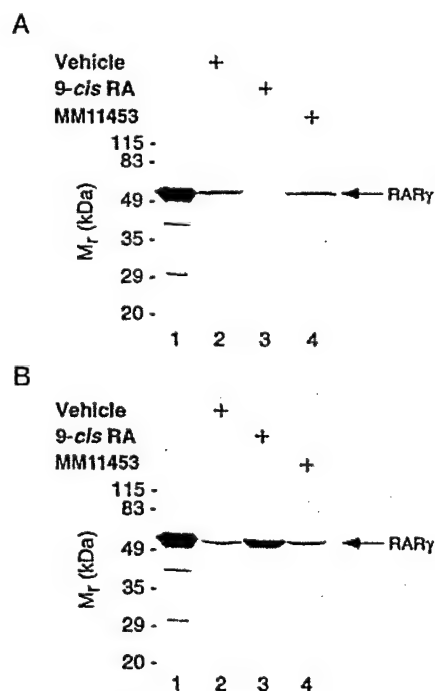


Fig. 6. MM11453 does not induce NCoR corepressor dissociation from RARγ or coactivator p300 recruitment to RARγ. A, NCoR-RARγ dissociation using [^{35}S]Met-RARγ and GST-NCoR 2110–2453 is as described in "Materials and Methods." Only 9-*cis*-RA induced NCoR-RARγ dissociation (Lane 3). In B, RARγ coactivator recruitment using GST-p300 (1–450) and [^{35}S]Met-RARγ is as in "Materials and Methods." Only 9-*cis*-RA enhanced binding of p300 to RARγ. Lane 1 in A and B represents $\sim 15\%$ of [^{35}S]Met-RARγ. Left, marker migration (molecular mass).

⁵V. J. Peterson, M. I. Deinzer, M. I. Dawson, K.-C. Feng, A. Fields, and M. Leid. Mass spectrometric analysis of agonist-induced retinoic acid receptor γ conformational change, unpublished results.

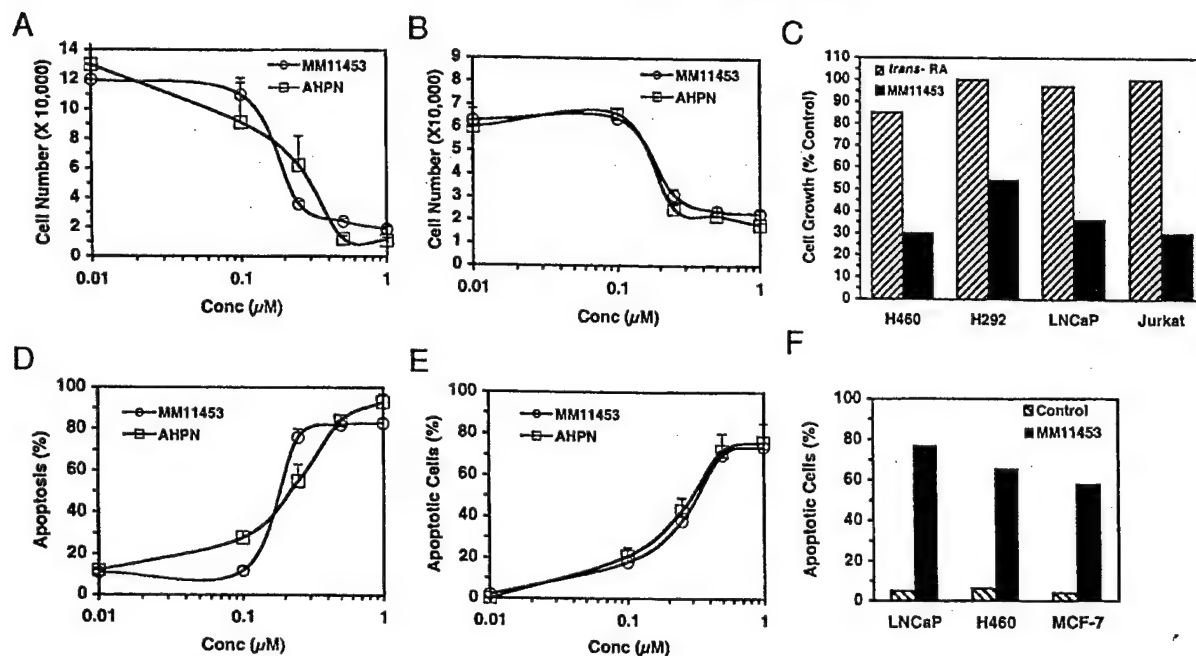


Fig. 7. MM11453 inhibits cell growth and induces apoptosis. HL-60R cells (A and D) and MDA-MB-231 cells (B and E) were treated with 10 nM to 1.0 μM MM11453, AHPN, or Me₂SO alone for 24 h and 120 h, respectively, as described in "Materials and Methods," and then harvested and counted (A and B) or assayed for apoptosis (D and E) as in "Materials and Methods." The results shown represent the means of three replicates; bars, SE. In C and F, H460, H292, LNCaP, and Jurkat cells were treated with ethanol alone, 1.0 μM *trans*-RA, or MM11453 for 48 h before viability was determined (C) or treated with 1.0 μM MM11453 or ethanol alone for 48 h before nuclear morphology was analyzed (F) as in "Materials and Methods." The experiments shown are representative of three replicates.

was $75 \pm 1\%$ and $76 \pm 7\%$, respectively. Thus, both MM11453 and AHPN are similarly apoptotic in retinoid-resistant cells. MM11453 at 1.0 μM induced apoptosis in LNCaP (38%), H460 (47%), and MCF-7 (57%) cells, as demonstrated by nuclear morphological changes (Fig. 7F). In other experiments using these cells, 1.0 μM AHPN was found to induce 21 (21), 37, and 42% apoptosis, respectively (data not shown). Thus, both MM11453 and AHPN also induce apoptosis in retinoid-sensitive cells.

MM11453 Induced TR3 Expression. TR3 expression must be induced for AHPN to cause lung cancer cell apoptosis (21). To determine whether 1.0 μM MM11453 had this capability, H460 and LNCaP cells were treated for 6 h. MM11453 strongly induced TR3 expression, whereas *trans*-RA did not (Fig. 8).

MM11453 Induced TR3 Mitochondrial Targeting. MM11453 at 1.0 μM induced the migration of transiently expressed TR3/ΔBDB-GFP to mitochondria in H460 cells, as indicated in Fig. 9 by colocalization of GFP fluorescence with that of immunostained Hsp60. Colocalization did not occur in vehicle-alone-treated cells (data not shown). Thus, both AHPN and MM11453 induce TR3 targeting to mitochondria.

MM11453 Altered Mitochondrial Membrane Potential. We found that MM11453 induced TR3 targeting to the mitochondrial outer membrane of breast and prostate cancer cells to initiate cytochrome *c* release and apoptosis (27). A loss of inner mitochondrial membrane potential or depolarization, which may signify outer membrane or permeability transition pore opening (45) and has been suggested as causing cytochrome *c* release (45), is associated with apoptosis. The effect of MM11453 on this process was explored using Rh123, which cells incorporate on depolarization. MM11453 increased MCF-7, MDA-MB-231, and LNCaP cell Rh123 fluorescence 2.2-, 1.9-, and 5.4-fold, respectively (Fig. 10). Again, MM11453 behaves similarly to AHPN (46).

Bcl-2 Attenuated Mitochondrial Membrane Depolarization by MM11453. Because overexpression of antiapoptotic, mitochondrial membrane-surface protein Bcl-2 is reported to block cancer cell apoptosis (47), its effect on apoptosis by MM11453 was explored in Jurkat cells transfected with an expression vector containing *bcl-2* or the vector alone. In MM11453-treated nontransfected cells and MM11453-treated vector alone-transfected cells, depolarized cell numbers increased 4.8- and 5.5-fold, respectively, over that of the nontreated control, whereas cell numbers increased only 2-fold in treated cells overexpressing *bcl-2* (Fig. 11). Thus, *bcl-2* modified the effect of MM11453 on mitochondrial membranes.

DISCUSSION

AHPN induces apoptosis in cancer cell lines (13, 14, 18–22, 24). How AHPN initiates this process remains to be completely defined. A report of the RARγ selectivity of AHPN (12) led to the hypothesis of an apoptotic role for RARγ in breast cancer, melanoma, and neuroblastoma cells (17, 23, 24). To support this, RARγ transcriptionally active AHPN derivatives and analogues were also reported to inhibit growth and induce apoptosis (28, 48, 49). Other reports present data strongly suggesting an RAR-independent pathway, such as growth inhibition and apoptosis of retinoid-resistant cancer cells (13, 14, 21, 27, 42, 44, 50).⁵ Our results support the latter by showing that MM11453, although unable to activate retinoid receptors on a reporter with the efficacy of standard retinoids or AHPN, strongly inhibited growth and induced apoptosis in retinoid-resistant cancer cell lines.

The near absence of RAR subtype and RXRα transcriptional activation by MM11453 was confirmed by limited proteolysis. DPSAs suggest that MM11453 is not a RAR or RXRα agonist. MM11453 did not induce a protease-resistant RARγ conformation, characteristic of binding a retinoid agonist, such as MM11254 [6], but behaved as the

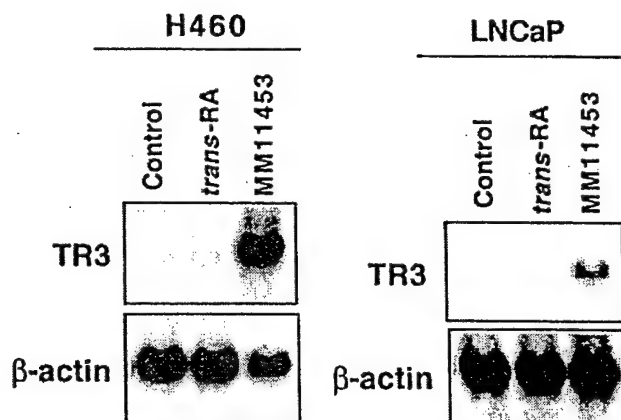


Fig. 8. MM11453 induces TR3 expression in H460 and LNCaP cells. Cells were treated with ethanol alone, 1.0 μ M *trans*-RA, or MM11453 for 6 h. Total RNAs were prepared and analyzed for TR3 expression by Northern blotting. Expression of β -actin was the RNA-loading control.

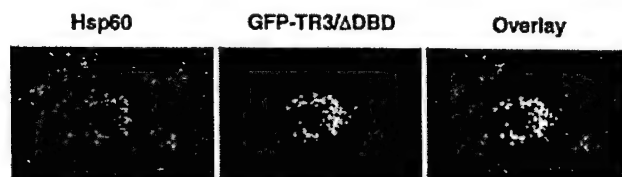


Fig. 9. MM11453 induces TR3 translocation to H460 mitochondria. Cells were transiently transfected with TR3/ΔDBD-GFP expression vector and then treated with 1.0 μ M MM11453 for 6 h as in "Materials and Methods." Immunostained mitochondrial Hsp60 (red) and TR3/ΔDBD-GFP protein (green) were visualized by confocal microscopy, and images were overlaid (Overlay) to indicate colocalization (yellow).

RAR γ -selective antagonist MM11253 [7], a dithiane (14, 39) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid].⁵ MM11453 did not detectably dissociate NCoR from RAR γ or recruit p300 to RAR γ , as agonists did. Thus, the behavior of MM11453 contrasts with that of RAR-agonist AHPN (12, 14). A retinoid receptor-independent pathway for anticancer activity has precedent in the mechanism of action of *N*-(4-hydroxy)phenyl retinamide, which inhibits the growth of cancer cells that resist standard retinoids (51, 52).

DPSA (data not shown) and molecular modeling (Fig. 2) suggest that AHPN does not induce a unique conformation in the RAR γ LBD that could account for apoptosis-inducing activity. RAR γ on binding AHPN, *trans*-RA, or MM11254 produced the same PFs,⁵ whereas 1.0 μ M MM11253 [7] did not induce this conformation⁵ or transcriptionally activate RAR γ (14). Both transactivation and DPSA show that the bulky 1-adamantyl group of AHPN (Fig. 2A) did not prevent an agonist-induced RAR γ conformation, and modeling shows the 1-adamantyl group occupying the same region as the tetrahydronaphthalene rings of agonists, MM11254 and BMS270394 [8] (Ref. 41; Fig. 2B). The three hydroxyl and carboxyl oxygen molecules are also close. Thus, on the basis of the strategy used by Klaholz *et al.* (41) that the low-energy conformation of a ligand approximates its bound form, our findings suggest that pharmacophoric AHPN groups are not responsible for inducing any unique conformation in RAR γ . Only the 3-chloro group *ortho* to the COOH group distinguishes MM11453 from AHPN. How the chloro group inhibits transcriptional activation remains to be determined. Both its steric and electronic properties may perturb hydrogen bonding by the COOH group or shift van der Waals contacts of RAR γ LBD pendant groups, thereby preventing the con-

formational changes in the receptor necessary for coactivator recruitment and transcriptional activation.

The inhibition of [³H]9-*cis*-RA binding to RARs by MM11453 suggests direct binding, whereas transfection indicates minimal RAR or RXR α agonism. Thus, MM11453 may function as a moderately selective RAR γ antagonist. Although how RAR γ antagonism or that of another RAR or RXR subtype contributes to MM11453 activity remains to be defined, the lack of growth inhibition by antagonist MM11253 (data not shown) suggests that the contribution, if any, is small. Unlike *trans*-RA, both MM11453 and AHPN strongly inhibited HL-60R, MDA-MB-231, LNCaP, and H292 cell growth and induced apoptosis. EC₅₀s for inhibiting growth in HL-60R and MDA-MB-231 cells were comparable, and their apoptotic EC₅₀s were similar (Fig. 7). These results indicate that MM11453 functions independently of RARs and RXR α and strongly suggest a similar mode of action for AHPN. Both AHPN (21) and MM11453 (Fig. 8) induced TR3 expression in H460 and LNCaP cells and TR3 mitochondrial translocation (Ref. 27 and Fig. 9, respectively) and caused inner mitochondrial membrane depolarization in MCF-7, MDA-MB-231, LNCaP, and Jurkat cells (Figs. 10 and 11). These results demonstrate that

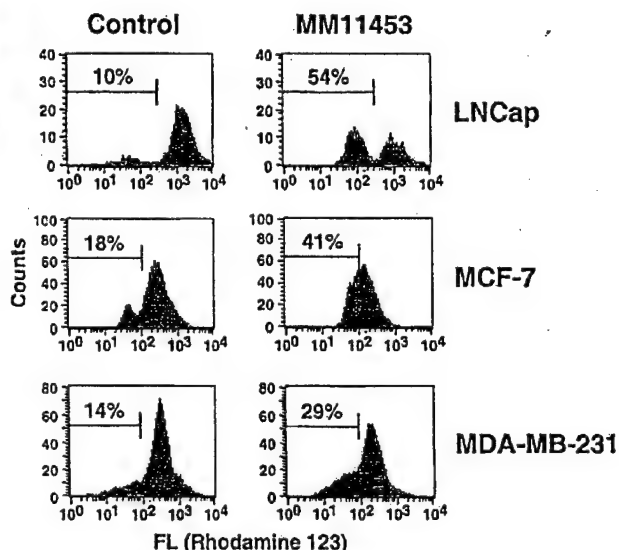


Fig. 10. Effect of MM11453 on LNCaP, MCF-7, and MDA-MB-231 mitochondrial membrane potential. Cells were treated with or without 1.0 μ M MM11453 for 18 h and then with Rh123 as in "Materials and Methods." Rh123-fluorescing cells are expressed as a percentage of the total.

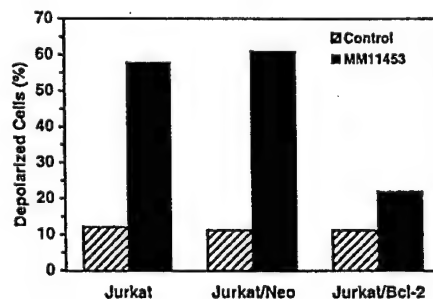


Fig. 11. Bcl-2 inhibits Jurkat mitochondrial membrane potential decrease by MM11453. Nontransfected cells stably expressing vector alone (*Jurkat/Neo*) and transfected cells stably expressing Bcl-2 (*Jurkat/Bcl-2*) were treated with 1.0 μ M MM11453 or ethanol alone for 18 h and analyzed for change in mitochondrial membrane potential as in "Materials and Methods."

MM11453 retains the apoptotic properties of AHPN without behaving as a competent RAR γ agonist and, thus, indicate that RAR γ activation is not required for apoptotic activity. The recent report that AHPN induces apoptosis in RAR γ -negative myeloma cells through a mitochondrial pathway (46) supports this conclusion. Reporter and limited proteolysis assays on MM11453 and AHPN suggest that their apoptotic activity does not involve RAR α , RAR β , or RXR α activation.

Transactivation by liganded RAR γ is reported to correlate with retinoid toxicity (53, 54). The lack of retinoid receptor activation activity by MM11453 suggests that toxic side effects characteristic of retinoid receptor activation (reviewed in Ref. 11) should be reduced in this class of apoptosis inducers, thereby affording more effective candidates for development as cancer chemotherapeutic agents.

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REFERENCES

- Nagpal, S., and Chandraratna, R. A. S. Recent developments in receptor-selective retinoids. *Curr. Pharm. Des.*, 6: 919-931, 2000.
- Mangelsdorf, D. J., Umesono, K., and Evans, R. M. The retinoid receptors. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids. Biology, Chemistry, and Medicine*, pp. 319-349. New York: Raven Press, Ltd., 1994.
- Salbert, G., Fanjul, A., Piedrafita, F. J., Lu, X.-P., Kim, S. J., Tran, P., and Pfahl, M. Retinoid acid receptors and retinoid X receptor- α down-regulate the transforming growth factor- β 1 promoter by antagonizing AP-1 activity. *Mol. Endocrinol.*, 7: 1347-1356, 1993.
- Caelles, C., Gonzalez-Sanchez, J. M., and Munoz, A. Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev.*, 11: 3351-3364, 1997.
- Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Spodisstrom, M., and Glass, C. K. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor corepressor. *Nature (Lond.)*, 377: 397-404, 1995.
- Li, X.-S., Shao, Z.-M., Sheikh, M. S., Eisman, J. L., Sentz, D., Jettin, A. M., Chen, J.-C., Dawson, M. I., and Fontana, J. Retinoid acid nuclear receptor β (RAR β) inhibits breast carcinoma growth and tumorigenicity. *J. Cell. Physiol.*, 16: 449-458, 1995.
- Liu, Y., Lee, M.-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J., and Zhang, X. RAR β mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol. Cell. Biol.*, 16: 1138-1149, 1996.
- Widshwendter, M., Berger, J., Hermann, M., Muller, H. M., Amberger, A., Zeschig, M., Widschwendter, A., Abendstein, B., Zeimet, A. G., Daxenbichler, G., and Marth, C. Methylation and silencing of the retinoic acid receptor- β 2 gene in breast cancer. *J. Natl. Cancer Inst. (Bethesda)*, 92: 826-832, 2000.
- Lin, B., Chen, G., Xiao, D., Kolluri, S., Cao, X., Li, H., Kin, F., Ran, R., Su, H., and Zhang, X. Orphan receptor COUP-TF is required for RAR β induction, growth inhibition, and apoptosis by retinoic acid in cancer cells. *Mol. Cell. Biol.*, 20: 957-970, 2000.
- Armstrong, R. B., Ashenfelter, K. O., Eckhoff, C., Levin, A. A., and Shapiro, S. S. General and reproductive toxicology of retinoids. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids. Biology, Chemistry, and Medicine*, pp. 545-572. New York: Raven Press, Ltd., 1994.
- Dawson, M. I., Zhang, X., Hobbs, P. D., and Jong, L. Synthetic retinoids and their usefulness in biology and medicine. In: M. A. Livrea (ed.), *Vitamin A and Retinoids: An Update of Biological Aspects and Clinical Applications*, pp. 161-196. Basel: Birkhäuser Verlag, 2000.
- Bernard, B. A., Bernardon, J. M., Delescluse, C., Martin, B., Lenoir, M. C., Maignan, J., Charpentier, B., Pilgrim, W. R., Reichert, U., and Shroot, B. Identification of synthetic retinoids with selectivity for human nuclear retinoic acid receptor β . *Biochem. Biophys. Res. Commun.*, 186: 977-983, 1992.
- Shao, Z. M., Dawson, M. I., Li, X. S., Rishi, A. K., Sheikh, M. S., Han, Q. X., Ordonez, J. V., Shroot, B., and Fontana, J. A. p53-independent G $_0$ /G $_1$ arrest and apoptosis induced by a novel retinoid in human breast cancer cells. *Oncogene*, 11: 493-504, 1995.
- Chao, W.-R., Hobbs, P. D., Jong, L., Zhang, X.-K., Zheng, Y., Wu, Q., Shroot, B., and Dawson, M. I. Effects of receptor class- and subtype-selective retinoids and an apoptosis-inducing retinoid on the adherent growth of the NIH:OVCAR-3 ovarian cancer cell line in culture. *Cancer Lett.*, 115: 1-7, 1997.
- Rishi, A. K., Sun, R.-J., Gao, Y., Hsu, C. K. A., Gerald, T. M., Sheikh, M. S., Dawson, M. I., Reichert, U., Shroot, B., Fornace, A. J., Jr., Brewer, G., and Fontana, J. A. Posttranscriptional regulation of the DNA damage-inducible *gadd45* gene in human breast carcinoma cells exposed to a novel retinoid. *Nucleic Acids Res.*, 27: 3111-3119, 1999.
- Tarapore, P., and Fukasawa, K. p53 mutation and mitotic infidelity. *Cancer Invest.*, 18: 148-155, 2000.
- Widschwendter, M., Daxenbichler, G., Culig, Z., Michel, S., Zeimet, A. G., Mörtl, M. G., Widschwendter, A., and Marth, C. Activity of retinoic acid receptor- γ selectively binding retinoids alone and in combination with interferon- γ in breast cancer cell lines. *Int. J. Cancer*, 71: 497-504, 1997.
- Oridate, N., Higuchi, M., Suzuki, S., Shroot, B., Hong, W. K., and Lotan, R. Rapid induction of apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (CD437). *Int. J. Cancer*, 70: 484-487, 1997.
- Hsu, S.-L., Yin, S.-C., Liu, M.-C., Reichert, U., and Ho, W. L. Involvement of cyclin-dependent kinase activities in CD437-induced apoptosis. *Exp. Cell Res.*, 252: 332-341, 1999.
- Hsu, C. A., Rishi, A. K., Li, X. S., Gerald, T. M., Dawson, M. I., Schiffer, C., Reichert, U., Shroot, B., Poirer, G. C., and Fontana, J. A. Retinoid-induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. *Blood*, 89: 4470-4479, 1997.
- Li, Y., Lin, B., Agadir, A., Liu, R., Dawson, M. I., Reed, J. C., and Zhang, X. Molecular determinants of AHPN (CD437)-induced growth arrest and apoptosis in human lung cancer cell lines. *Mol. Cell. Biol.*, 18: 4719-4731, 1998.
- Adachi, H., Adams, A., Hughes, F. M., Zhang, J., Cidlowski, J. A., and Jetten, A. M. Induction of apoptosis by the novel retinoid AHPN in human T-cell lymphoma cells involves caspase-dependent and -independent pathways. *Cell Death Differ.*, 5: 973-983, 1998.
- Schadenförf, D., Worm, M., Jurgovski, K., Dippel, E., Michel, S., Charpentier, B., Bernardon, J. M., Reichert, U., and Czarnetzki, B. M. Retinoic acid receptor γ -selective retinoids exert antiproliferative effects on human melanoma cell growth *in vitro*. *Int. J. Oncol.*, 5: 1325-1331, 1994.
- Meister, B., Fink, F.-M., Hittmair, A., Marth, C., and Widschwendter, M. Antiproliferative activity and apoptosis induced by retinoic acid receptor- γ selectively binding retinoids in neuroblastoma. *Anticancer Res.*, 18: 1777-1786, 1998.
- Liang, J.-Y., Fontana, J. A., Rao, J. N., Ordonez, J. V., Dawson, M. I., Shroot, B., Wilber, J. F., and Feng, P. A synthetic retinoid CD437 induces S-phase arrest and apoptosis. *Prostate*, 38: 228-236, 1999.
- Robertson, K. A., Emami, B., and Collins, S. J. Retinoic acid-resistant HL-60R cells harbor a point mutation in the retinoic acid receptor ligand-binding domain that confers dominant negative activity. *Blood*, 81: 1881-1887, 1992.
- Li, H., Kolluri, S. K., Gu, J., Dawson, M. I., Cao, X., Hobbs, P. D., Lin, B., Chen, G. L. J., Lin, F., Xie, Z., Fontana, J. A., Reed, J. C., and Zhang, X. Cytochrome c release and apoptosis induced by mitochondrial targeting of orphan receptor TR3 (nurf77, NGFI-B). *Science (Wash. DC)*, 289: 1159-1164, 2000.
- Charpentier, B., Bernardon, J.-M., Eustache, J., Millois, C., Martin, B., Michel, S., and Shroot, B. Synthesis, structure-affinity relationships and biological activities of ligands binding to retinoic acid receptor subtypes. *J. Med. Chem.*, 38: 4993-5006, 1995.
- Sakashita, A., Kizaki, M., Pakkala, S., Schiller, G., Tsuruoka, N., Tomosaki, R., Cameron, J. F., Dawson, M. I., and Koeffler, H. P. 9-*cis*-Retinoic acid: effects on normal and leukemic hematopoiesis *in vitro*. *Blood*, 81: 1009-1016, 1993.
- Zhang, X., Hoffmann, B., Tran, P., Graupner, G., and Pfahl, M. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature (Lond.)*, 355: 441-446, 1992.
- Wu, Q., Dawson, M. I., Zheng, Y., Hobbs, P. D., Agadir, A., Jong, L., Li, Y., Liu, R., Lin, B., and Zhang, X. Inhibition of *trans*-RA-resistant human breast cancer cell growth by retinoid X-receptor-selective retinoids. *Mol. Cell. Biol.*, 17: 6598-6608, 1997.
- Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S., and Chambon, P. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell*, 68: 377-395, 1992.
- Minucci, S., Leid, M., Toyama, R., Saint-Jeannet, J.-P., Peterson, V. J., Horn, V., Ishmael, J. E., Bhattacharyya, N., Dey, A., David, I. B., and Ozato, K. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid dependent gene expression. *Mol. Cell. Biol.*, 17: 644-655, 1997.
- Leid, M. Ligand-induced alteration of the protease sensitivity of retinoid X receptor. *J. Biol. Chem.*, 269: 14175-14181, 1994.
- Dowell, P., Ishmael, J., Avram, D., Peterson, V., Nevriy, D., and Leid, M. p300 functions as a coactivator for the peroxisome proliferator-activated receptor α . *J. Biol. Chem.*, 272: 33435-33443, 1997.
- Dowell, P., Ishmael, J. E., Avram, D., Peterson, V. J., Nevriy, D. J., and Leid, M. Identification of nuclear receptor corepressor as a peroxisome proliferator-activated receptor α -interacting protein. *J. Biol. Chem.*, 274: 15901-15907, 1999.
- Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. Bcl-2 $_L$ regulates the membrane potential and volume homeostasis of mitochondria. *Cell*, 91: 627-637, 1997.
- Miyashi, T., Kitada, S., Krajewski, S., Horne, W. A., Delia, D., and Reed, J. C. Overexpression of the Bcl-2 protein increases the half-life of p21^{Bax}. *J. Biol. Chem.*, 270: 26049-26052, 1995.
- Dawson, M. I., Chao, W., Pine, P., Jong, L., Hobbs, P. D., Rudd, C., Quick, T. C., Niles, R. M., Zhang, X., Lombardo, A., Ely, K. R., Shroot, B., and Fontana, J. A. Correlation of retinoid binding affinity to RAR α with retinoid inhibition of growth of estrogen receptor-positive MCF-7 mammary carcinoma cells. *Cancer Res.*, 55: 4446-4451, 1995.

40. Klaholz, B. P., Mitschler, A., and Moras, D. Structural basis for isotype selectivity of the human retinoic acid receptor. *J. Mol. Biol.*, 302: 155-170, 2000.
41. Klaholz, B. P., Mitschler, A., Belema, M., Zusi, C., and Moras, D. Enantiomer discrimination illustrated by high-resolution crystal structures of the human nuclear receptor hRAR γ . *Proc. Natl. Acad. Sci. USA*, 97: 6322-6327, 2000.
42. Graupner, G., Malle, G., Maignan, J., Lang, G., Prunieras, M., and Pfahl, M. 6'-Substituted naphthalene-2-carboxylic acid analogs, a new class of retinoic acid receptor subtype-specific ligands. *Biochem. Biophys. Res. Commun.*, 179: 1554-1561, 1991.
43. Li, X.-S., Rishi, A. K., Shao, Z.-M., Dawson, M. I., Jong, L., Shroot, B., Reichert, U., Ordenez, J., and Fontana, J. A. Posttranscriptional regulation of p21^{WAF-1/CIP-1} expression in human breast carcinoma cells. *Cancer Res.*, 56: 5055-5062, 1996.
44. Fontana, J. A., Dawson, M. I., Leid, M., Rishi, A. K., Zhang, Y., Hsu, C.-A., Lu, J. S., Peterson, V. J., Jong, L., Hobbs, P., Chao, W.-R., Shroot, B., and Reichert, U. Identification of a unique binding protein for a novel retinoid inducing cellular apoptosis. *Int. J. Cancer*, 86: 474-479, 2000.
45. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. *Science (Wash. DC)*, 281: 1309-1312, 1998.
46. Marchetti, P., Zamzami, N., Joseph, B., Schraen-Maschke, S., Mereau-Richard, C., Costantini, P., Metivier, D., Susin, S. A., Kroemer, G., and Formstecher, P. The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. *Cancer Res.*, 59: 6257-6266, 1999.
47. Reed, J. C. Bcl-2 family proteins. *Oncogene*, 17: 3225-3236, 1998.
48. Lu, X. P., Fanjul, A., Picard, N., Shroot, B., and Pfahl, M. A selective retinoid with high activity against an androgen-resistant prostate cancer cell type. *Int. J. Cancer*, 80: 272-278, 1999.
49. Lu, X. P., Fanjul, A., Picard, N., Pfahl, M., Rungta, D., Nared-Hook, K., Carter, B., Piedrafita, J., Tang, S., Fabbizio, E., and Pfahl, M. Novel retinoid-related molecules as apoptosis inducers and effective inhibitors of human lung cancer cells *in vivo*. *Nat. Med.*, 3: 686-690, 1997.
50. Sun, S. Y., Yue, P., and Lotan, R. Implications of multiple mechanisms in apoptosis induced by the synthetic retinoid CD437 in human prostate carcinoma cell. *Oncogene*, 19: 4513-4522, 2000.
51. Delia, D., Aiello, A., Lombardi, L., Pelicci, P. G., Grignani, F., Grignani, F., Formelli, F., Menard, S., Costa, A., Veronesi, U., and Pierotti, M. A. *N*-(4-Hydroxyphenyl)-retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. *Cancer Res.*, 53: 6036-6041, 1993.
52. Sheikh, M. S., Shao, Z.-M., Li, X.-S., Ordenez, J. V., Conley, B. A., Wu, S., Dawson, M. I., Han, Q.-X., Chao, W., Quick, T., Niles, R. M., and Fontana, J. A. *N*-(4-Hydroxyphenyl)-retinamide (4-HPR)-mediated biological actions involve retinoid receptor-independent pathways in human breast carcinoma. *Carcinogenesis (Lond.)*, 16: 2477-2486, 1995.
53. Reczek, P. R., Ostrowski, J., Yu, K. L., Chen, S., Hammer, L., Roalsvig, T., Starrett, J. E., Jr., Driscoll, J. P., Whiting, G., Spinazze, P. G., Tramposch, K. M., and Mansuri, M. M. Role of retinoic acid receptor γ in the Rhino mouse and rabbit irritation models of retinoidal activity. *Skin Pharmacol.*, 8: 292-299, 1995.
54. Standeven, A. M., Teng, M., and Chandraratna, R. A. S. Lack of involvement of retinoic acid receptor α in retinoid-induced skin irritation in hairless mice. *Toxicol. Lett.*, 92: 231-240, 1997.

Vitamin A and apoptosis in prostate cancer

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Abstract

Apoptosis represents an effective way to eliminate cancer cells. Unfortunately, advanced prostate tumors eventually progress to androgen-independent tumors, which are resistant to current therapeutic approaches that act by triggering apoptosis. Vitamin A and its natural and synthetic analogs (retinoids) induce apoptosis in prostate cancer cells *in vitro* and in animal models, mainly through induction of retinoic acid receptor- β (RAR β). Expression levels of RAR β , however, are significantly reduced in hormone-independent prostate cancer cells. Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) ^{that} effectively induces apoptosis of both hormone-dependent and -independent prostate cancer cells in a retinoid receptor-independent manner. ^{was identified and has drawn a lot of attention in the field} The apoptotic effect of AHPN requires expression of orphan receptor TR3 (also called nur77 or NGFI-B). Paradoxically, TR3 expression is also induced by androgen and other mitogenic agents in prostate cancer cells to confer their proliferation. The recent finding that TR3 migrates from the nucleus to mitochondria to trigger apoptosis in response to AHPN suggests that the opposing biological activities of TR3 are regulated by its subcellular localization. Thus, agents that induce translocation of TR3 from the nucleus to mitochondria will have improved efficacy against prostate cancer. TR3, therefore, represents an unexplored molecule that may be an ideal target for developing new agents for prostate cancer therapy.

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Introduction

Prostate cancer is the most common cancer diagnosed among men in the United States, accounting for 27.5% of all cancer cases in men. It ranks second after lung cancer as the underlying cause of cancer death in US men. Despite aggressive efforts toward earlier detection and treatment, the mortality rate for prostatic carcinoma has steadily increased. The identification of androgens as the major regulator of prostatic epithelial proliferation offered a target for therapeutic intervention. Androgen ablation by surgical gonadectomy or drug treatments that suppress androgen production and action remain the only effective form of therapy for men with advanced disease. Unfortunately, the median duration of response to androgen ablation is less than 2 years, after which the disease will re-emerge in a poorly differentiated, androgen-independent form, which is often fatal. The lack of therapies for this advanced prostate cancer has contributed significantly to the increased mortality rates, and has resulted in the impetus to develop non-androgen-based therapies.

Vitamin A and its natural and synthetic analogs, retinoids, are one of the most investigated classes of chemopreventive drugs for prostate cancer. Early experiments on mouse prostate explant cultures showed that all-*trans*-

retinoic acid (*trans*-RA) could both inhibit and reverse the proliferative effects of chemical carcinogens on prostatic epithelium (Lasnitzki & Goodman 1974, Chopra & Wilkoff 1976). Recent studies have demonstrated that retinoids effectively inhibit the growth of prostate cancer cells *in vitro* and suppress the development of prostate carcinogenesis (Blutt *et al.* 1997, DiPaola *et al.* 1997, Campbell *et al.* 1998, Goossens *et al.* 1999, McCormick *et al.* 1999, Pasquali *et al.* 1999, Richter *et al.* 1999, Sun *et al.* 1999b, Urban *et al.* 1999, Webber *et al.* 1999, Kelly *et al.* 2000, Koshiuka *et al.* 2000, Lotan *et al.* 2000, Tanabe 2000, Pili *et al.* 2001). Clinical trials of several retinoids and their combination with other anti-cancer agents have shown significant activities, ^{when retinoids were used in combination with other chemotherapeutic agents, such as interferon- α and paclitaxel} (DiPaola *et al.* 1997, 1999, Culine *et al.* 1999, Shalev *et al.* 2000, Thaller *et al.* 2000). Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) (Bernard *et al.* 1992) was found to potently inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate cancer cells. Thus, these small molecules may serve as prototypes for the

development of new prostate cancer therapeutic and preventive agents. The recent identification of the molecular targets of retinoid action in prostate cancer cells offers opportunities for the development of novel therapeutic strategies.

Vitamin A signaling pathways

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (Zhang *et al.* 1992b, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). RARs and RXRs are encoded by three distinct genes (α , β and γ). In addition, many retinoid receptor isoforms are generated through differential promoter usage, giving rise to a large number of distinct retinoid receptor proteins. To date, there are dozens of receptors which are known to mediate the effect of retinoids cloned. 9-*cis* RA is a high-affinity ligand for both RARs and RXRs, whereas *trans*-RA is a ligand for only RARs. Retinoid receptors belong to a large steroid/thyroid receptor superfamily that mediate the biological effects of many hormones, vitamins and drugs. RARs and RXR act as transcriptional factors to positively or negatively regulate expression of target genes by binding to their response elements (RAREs) located in promoter regions of the target genes (Fig. 1). The physiological role of RARs and RXRs has been extensively studied by knockout experiments (Kastner *et al.* 1995). Knockout of most of individual RARs activity by homologous recombination appears normal due to redundancy in the function of RARs *in vivo*. However, knockout of RAR α and RAR γ as well as RAR double knockouts produces defects that resemble the postnatal vitamin A-deficient syndrome and can be prevented by *trans*-RA administration, including keratinizing squamous metaplasia of the prostate gland (Kastner *et al.* 1995).

RXRs form heterodimers with many nuclear receptors including RARs, thyroid hormone receptor (TR), vitamin D receptor and peroxisome proliferator-activated receptor (PPAR) (Zhang *et al.* 1992b, Kastner *et al.* 1995, Mangelsdorf & Evans 1995), thereby mediating diverse endocrine signaling pathways. The function of RARs, however, is more restricted. The role of ligands in the regulation of retinoid receptor function is complex. RAR/RXR is activated mainly through binding of RAR with its ligand, although there are some situations where binding of both the RAR and RXR components with their respective ligands can contribute to the activity of the RAR/RXR heterodimers (Zhang *et al.* 1992b, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). The retinoid binding to RXRs is required for the activation of RXR homodimers and certain RXR heterodimers, such as TR3/RXR and PPAR γ /RXR (Zhang & Pfahl 1993, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). Unliganded retinoid receptors can act as negative transcription factors by binding to the RAREs of retinoid target genes, and recruit receptor corepressors, such as NcoR (Xu *et al.* 1999), leading to

histone deacetylation and formation of an inactive chromatin structure preventing transcription. Binding of retinoids to their receptors induces receptor conformational changes that serve as switches by releasing the receptor corepressors and by facilitating the recruitment of receptor co-activators, such as CBP (Xu *et al.* 1999) (Fig. 1). Several of the co-activator proteins have histone acetylase activity that contributes to the formation of an active chromatin structure and results in the transcription of target genes. (remove)

In addition to their direct effects on transcription, liganded RAR can modulate the activity of other transcriptional factors, such as AP-1 (Pfahl 1993). Activated retinoid receptors can inhibit the activity of AP-1, thereby regulating the expression of AP-1 target genes. The inhibition of AP-1 activity is linked to the anti-proliferative effects of retinoids, and appears to be separable from their direct activation of transcription of retinoid-target genes. Synthetic retinoids that specifically inhibit AP-1 activity without activating transcription have been developed (Fanjul *et al.* 1994, Chen *et al.* 1995, Li *et al.* 1996). These AP-1-specific retinoids can inhibit cell proliferation *in vitro*.

Recent evidence indicating that the cytoplasmic action of several hormone receptors represents an important mechanism for regulating their biological function has accumulated. The proapoptotic effect of the orphan receptor TR3 (also known as nur77 and NGFI-B) does not require its transcriptional regulation because TR3 with its DNA-binding domain deleted is still capable of inducing apoptosis (Li *et al.* 2000). In contrast, the cytoplasmic action of TR3, through its mitochondrial targeting, is essential for its apoptotic activity (Li *et al.* 2000). The glucocorticoid receptor was also found to reside on mitochondria (Scheller *et al.* 2000), while differentiation of PC12 pheochromocytoma cells is accompanied by nuclear export of NGFI-B (Katagiri *et al.* 2000). Estrogen receptors and androgen receptors trigger cell proliferation through their interaction with Src or phosphatidylinositol-3-OH kinase in the cytoplasm (Migliaccio *et al.* 2000, Simoncini *et al.* 2000, Kousteni 2001).

Apoptotic signalings

Apoptosis, also known as programmed cell death, is an evolutionarily conserved and indispensable process during normal embryonic development, tissue homeostasis and regulation of the immune system (Fisher 1994, Steller 1995, White 1996). The apoptotic process can be initiated by several different stimuli, including growth factor withdrawal, DNA damage, deregulation of the cell cycle or ligation of death receptors (Fisher 1994, Steller 1995, White 1996). These different apoptotic stimuli induce diverse early signaling events, which then converge by activating a common central biochemical pathway that is responsible for the execution of apoptosis. Execution of apoptosis is primarily mediated by caspases, a family of cysteine proteases with

8 Pfahl
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The receptor may be divided into five regions (A, B, C, D, E, and F) based on structure and function similarities among members of the steroid/thyroid hormone receptor superfamily.

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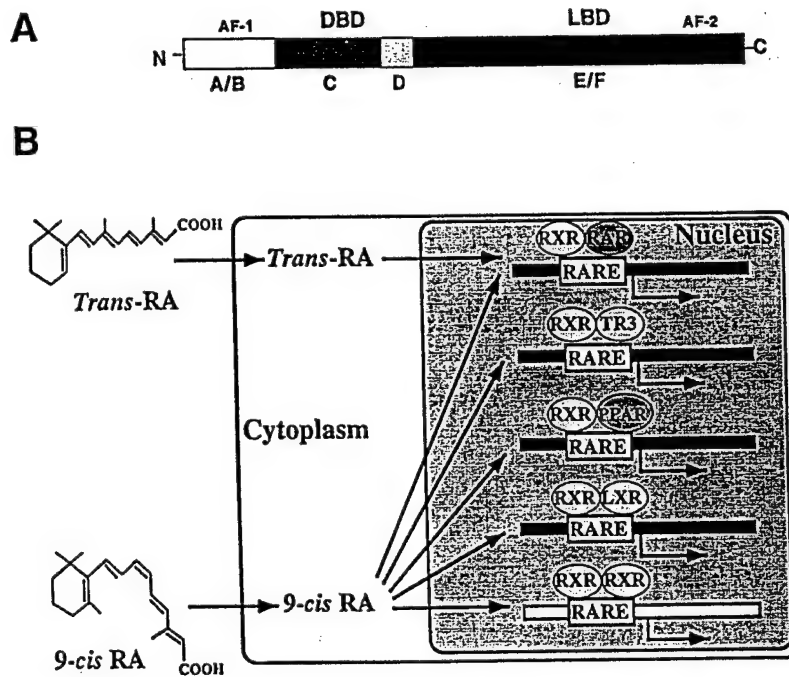


Figure 1 Retinoid signaling. Retinoid receptors are ligand-dependent transcription factors. (A) Schematic representation of retinoid receptor. DNA binding domain (DBD), ligand binding domain (LBD) and transactivation domains (AF-1 and AF-2) are indicated. (B) Mechanism of action of retinoid receptors. *Trans*-RA or 9-*cis* RA enter cells directly from the circulation, and bind to DNA-bound RAR or RXR, thereby eliciting a transcriptional response.

specificity for aspartic acid residues (Nunez *et al.* 1998, Thornberry & Lazebnik 1998).

There are two distinctly different pathways, the extrinsic and intrinsic pathways, transducing the death signals to caspase-mediated apoptotic machinery (Nunez *et al.* 1998). The extrinsic pathway involves activation of the superfamily of the tumor necrosis factor receptors (TNFR) or CD95 (Fas), by binding to their respective ligands, which in turn recruit procaspase-8 and -10 to membrane-associated signaling complexes, resulting in their activation (Fig. 2). Activation of these upstream caspases is sufficient to directly activate effector caspases such as caspase-3, -6 and -7, or indirectly induce apoptosis by cleaving Bid involved in the release of mitochondrial cytochrome c. The intrinsic pathway is activated directly by various forms of cellular stress that trigger mitochondrial release of cytochrome c into the cytosol. Cytosolic cytochrome c then binds to, and triggers oligomerization of the CED-4 homolog Apaf-1. The resulting 'apoptosome' recruits and activates procaspase-9 which, in turn, recruits and activates effector caspases, such as caspase-3 and possibly caspase-7 (Fig. 2). Additionally, the caspases can be activated by granzyme B, a major serine protease in cytotoxic lymphocyte granules (Shi *et al.* 1992). Once the effector caspases are activated, these enzymes cleave a number of cellular polypeptides leading to disassembly of key structural components of the nucleus and cytoskeleton,

inhibition of DNA repair, replication, and transcription, and activation of endonucleases that irreversibly damage the genome (Fisher 1994, White 1996).

Members of the Bcl-2 family are known to modulate apoptosis in different cell types in response to various stimuli (Adams & Cory 1998, Reed 1998). Some members act as antiapoptotic proteins, such as Bcl-2 and Bcl-XL, whereas others function as proapoptotic proteins, such as BAX and BAK. Proapoptotic and antiapoptotic members can heterodimerize and seemingly titrate one another's function. Many Bcl-2 family proteins reside on the mitochondrial outer membrane (Adams & Cory 1998, Reed 1998). Bcl-2 prevents mitochondrial disruption and the release of cytochrome c from mitochondria, while Bax and Bak create pores in mitochondria membranes and induce cytochrome c release. In addition, most proapoptotic proteins antagonize antiapoptotic proteins through heterodimerization with them (Adams & Cory 1998, Reed 1998). Caspase-dependent apoptosis can also be regulated by members of the inhibitors of apoptosis (IAP) protein family. IAPs suppress apoptosis by physically interacting with and inhibiting the catalytic activity of caspases (Deveraux & Reed 1999). In apoptotic cells, the caspase inhibition by IAPs is negatively regulated by a mitochondrial protein Smac/DIABLO, which is released from the mitochondrial intermembrane space into the cytosol upon apoptotic stimuli (Du *et al.* 2000, Verhagen *et al.* 2000).

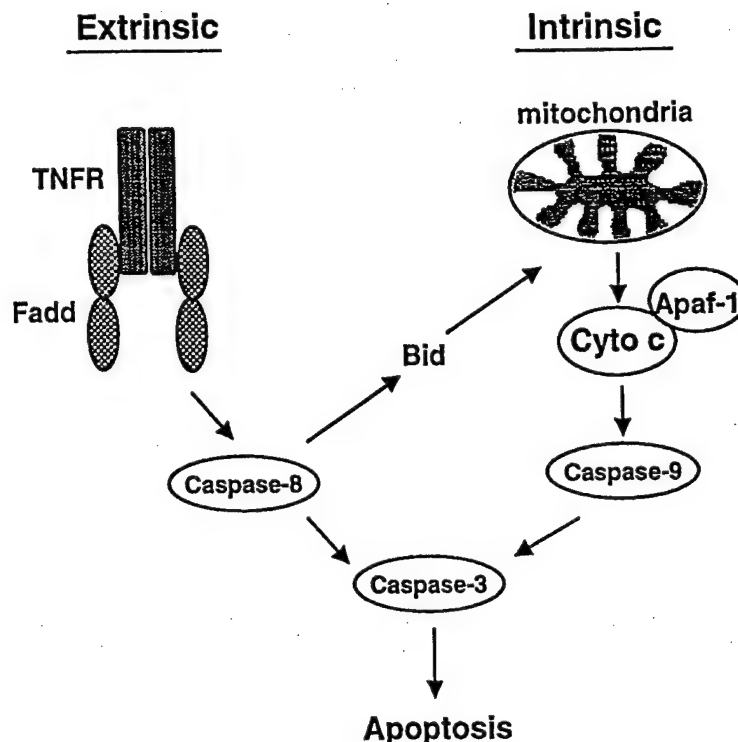


Figure 2 Apoptosis pathways. In the extrinsic pathway, ligation of death receptors activates initiator caspase-8 through the adaptor molecule Fadd. In the intrinsic pathway, cytochrome c (Cyto c) is released from mitochondria in response to a variety of death stimuli and binds to Apaf-1 to activate caspase-9. Active caspase-8 or -9 then activates effector caspases, such as caspase-3 resulting in morphological features of apoptosis. Caspase-8 also activates Bid, which then targets mitochondria to induce cytochrome c release, providing a link between the two pathways.

Apoptosis and prostate cancer development

Impaired apoptosis is involved in tumor initiation and progression, since apoptosis normally eliminates cells with increased malignant potential such as those with damaged DNA or aberrant cell cycling (Fisher 1994, Thompson 1995). Most prostate cancer cells have a protracted history of development, suggesting that prostate cancer cells must have evolved various mechanisms to subvert the apoptotic program (Bruckheimer & Kyprianou 2000). Impaired apoptosis signaling and extended cell survival seem to be closely associated with prostate tumor initiation, metastasis and progression to the androgen-insensitive state (Coffey *et al.* 2001). Increased levels of Bcl-2 are associated with emergence of an androgen-independent phenotype and overexpression of Bcl-2 can facilitate multistep prostate carcinogenesis in an animal model (Bruckheimer *et al.* 2000). Proapoptotic Bax contains a polymorphism in an unstable microsatellite causing a frameshift in androgen-independent DU145 cells (Rampino *et al.* 1997).

Recent studies have indicated a crucial role of the PTEN

tumor suppressor in the regulation of prostate cancer development. PTEN catalyzes dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate and antagonizes signaling pathways that rely on PI3K activity (Wu *et al.* 1998). PTEN is frequently inactivated in primary human prostate cancers, particularly in the more advanced cancers (Iltmann 1998), in human prostate xenografts and in cell lines (Li *et al.* 1997, Vlietstra *et al.* 1998, Whang *et al.* 1998). Release of the negative regulation of the PI3K pathway by PTEN may activate the cell survival kinase Akt during prostate tumor progression (Stambolic *et al.* 1998). Indeed, activated Akt regulates a number of intracellular events implicated in prostate tumor progression and androgen independence. Disruption of PTEN leads to suppression of apoptosis (Stambolic *et al.* 1998), due to inactivation of Bad (Datta *et al.* 1997) or caspase-9 (Cardone *et al.* 1998) by Akt. The disruption can also accelerate cell cycle progression (Sun *et al.* 1999a), through suppression of AFX/Forkhead transcription factor activity by Akt (Brunet *et al.* 1999, Kops *et al.* 1999), resulting in inhibition of cell cycle inhibitor p27 expression (Medema *et al.* 2000). The central role played by PTEN has been recently confirmed by the finding that mice with double mutants

PTEN(+)/(–) p27(–)/(–) develop prostate cancer at complete penetrance within 3 months from birth (Di Cristofano *et al.* 2001).

Androgen ablation and apoptosis

Androgen withdrawal is the primary choice of therapy for men with advanced prostate cancer, and it generally leads to regression of the disease. It is believed that apoptosis is mainly responsible for the regression of prostate cancer cells (Buttayan *et al.* 2000) and increased levels of apoptosis were indeed observed in human prostate cancer cells after androgen withdrawal (Denmeade *et al.* 1996, Reuter 1997, Montironi *et al.* 1998). However, in the CWR22 human prostate cancer xenograft model it was shown that the regression was due to cell cycle arrest rather than to apoptosis (Agus *et al.* 1999). It remains to be further investigated as to what degree that apoptosis is involved in tumor regression and how the process is regulated.

Progression to androgen independence after androgen-deprivation therapy is a multifactorial process by which cells acquire the ability to proliferate in the absence of androgens. Altered expression of apoptotic-regulatory genes likely plays some role in the development of hormone resistance of prostate cancer (Howell 2000). In the LNCaP prostate tumor model, adjuvant treatment with antisense Bcl-2 oligonucleotides after castration delays progression to androgen independence (Gleave *et al.* 1999). Androgen-independent prostate cancer cells also show resistance to apoptosis induction by chemotherapeutic agents and radiotherapy (Bruckheimer & Kyprianou 2000, Szostak & Kyprianou 2000). Overexpression of Bcl-2 and Bcl-XL is found in many androgen-independent cell lines and may be responsible for resistance to apoptosis (Bruckheimer & Kyprianou 2000, Coffey *et al.* 2001, Li *et al.* 2001), and antisense Bcl-2 oligonucleotides sensitize prostate cancer cells to the apoptotic effect of chemotherapeutic agents (Leung *et al.* 2001).

Retinoids and prostate cancer apoptosis

Growing evidence suggests that induction of apoptosis is a major mode of cell death in response to most cancer chemopreventive and chemotherapeutic agents (Fisher 1994, Thompson 1995, Bruckheimer & Kyprianou 2000). Retinoids exert potent apoptotic effects both in development and in cancer cells (Nagy *et al.* 1998). Retinoid-induced teratogenesis is associated with craniofacial malformations due to excessive apoptosis in the region (Sulik *et al.* 1988), while the limb malformations induced by retinoids are also associated with excessive cell death in the apical ectodermal ridge (Sulik & Dehart 1988). Retinoids regulate the development of the central nervous system in part through its apoptotic effect (Alles & Sulik 1990, 1992).

Induction of apoptosis by retinoids has been observed in various prostate cancer cells *in vitro* and *in vivo*. *Trans*-RA induces apoptosis of normal and malignant epithelial prostate cells (Pasquali *et al.* 1999), and it strongly enhances the apoptotic effect of docetaxel in DU-145 and LNCaP prostate cancer cells (Nehme *et al.* 2001). The combination of *trans*-RA and organic arsenical melarsoprol synergistically induces apoptosis of DU-145 and PC-3 cells *in vitro* and in immunodeficient mice (Koshiuka *et al.* 2000). The synthetic retinoid N-(4-hydroxyphenyl) retinamide (4HPR) is known to induce apoptosis in various malignant cells (Nagy *et al.* 1998). 4HPR also induces apoptosis of androgen-dependent and -independent cells (Sun *et al.* 1999b, Webber *et al.* 1999). The combination of 13-*cis* RA and phenylbutyrate synergistically induces apoptosis of several human and rodent prostate carcinoma cell lines (Pili *et al.* 2001).

The molecular mechanisms by which retinoids induce apoptosis of prostate cancer cells remain largely unknown. Induction of apoptosis of prostate cancer cells by several retinoids appears to be associated with down-regulation of Bcl-2 expression (DiPaola & Aisner 1999, DiPaola *et al.* 1999, Pasquali *et al.* 1999, Nehme *et al.* 2001), induction of insulin-like growth factor-binding protein-3 (IGFBP-3) (Goossens *et al.* 1999) and tissue transglutaminase (Pasquali *et al.* 1999), an enzyme that accumulates in cells undergoing apoptosis. Interestingly, RXR α was found to interact with IGFBP-3, and IGFBP-3-induced apoptosis was abolished in RXR α -knockout cells. It is likely that RXR α /IGFBP-3 interactions modulate the effects of IGFBP-3 on apoptosis (Liu *et al.* 2000).

RAR β and retinoid responses

The involvement of retinoid receptors in mediating proapoptotic effects of retinoids is complex, since some retinoids may act in a retinoid receptor-independent manner. However, many studies have suggested a crucial role of RAR β in the modulation of retinoid-induced apoptosis of prostate cancer cells. RAR β is up-regulated during apoptosis induced by the combination of phenylbutyrate and 13-*cis* RA in human and rodent prostate carcinoma cell lines and prostate tumors in the xenograft model (Pili *et al.* 2001), suggesting that RAR β expression may mediate the growth-inhibitory effect of retinoids. RAR β was also induced during *trans*-RA-induced apoptosis of prostate cancer cells (Pasquali *et al.* 1999). The expression of RAR β in 4HPR-treated prostate tissue was slightly higher than in the placebo-treated group (Lotan *et al.* 2000). Interestingly, introduction of RAR β in RAR β -negative prostate cancer cells resulted in increased sensitivity to the growth-inhibitory effect of retinoids and vitamin D (Campbell *et al.* 1998).

The role of RAR β in mediating the growth-inhibitory effect of retinoids was also demonstrated in many different

types of cancer cells, including breast, lung, ovarian, neuroblastoma, renal cell, pancreatic, liver, and head and neck (Nervi *et al.* 1991, Li *et al.* 1995, Hoffman *et al.* 1996, Liu *et al.* 1996, Kaiser *et al.* 1997, Xu *et al.* 1997b, Campbell *et al.* 1998, Ferrari *et al.* 1998, Li & Wan 1998). Expression of RAR β in RAR β -negative cancer cells restored *trans*-RA-induced growth inhibition and apoptosis, whereas inhibition of RAR β expression in RAR β -positive cancer cells abolished *trans*-RA effects (Li *et al.* 1995, Liu *et al.* 1996, Li & Wan 1998). In addition, transgenic mice expressing RAR β anti-sense sequences showed increased incidence of lung tumors (Berard *et al.* 1996), whereas suppression of RAR β expression was responsible for diminished anti-cancer activities of retinoids in animals (Wang *et al.* 1999). The expression of RAR β decreases as breast cells become progressively more malignant (Xu *et al.* 1997a), suggesting that loss of RAR β may lead to breast cancer development. Furthermore, up-regulation of RAR β is associated with a positive clinical response to retinoid in patients with premalignant oral lesions (Lotan *et al.* 1995).

The involvement of RAR β is also implicated by the finding that its expression mediates prostatic ductal branching morphogenesis in response to retinoids (Aboseif *et al.* 1997). However, expression was significantly reduced in malignant prostates compared with normal prostates (Lotan *et al.* 2000). In contrast, RAR α , RAR γ , RXR α and RXR γ were expressed in both normal and prostate tumor tissues (Lotan *et al.* 2000). RAR β was also selectively lost in DU-145 and PC-3 androgen-independent prostate cancer cells lines while RAR α , RAR γ and RXR α were well expressed (Campbell *et al.* 1998, Sun *et al.* 1999b). These observations suggest that loss of RAR β is associated with prostate carcinogenesis. The fact that reduced RAR β was observed in the normal tissue adjacent to the tumor suggests that this change is an early event in prostate carcinogenesis (Lotan *et al.* 2000). Similar changes were also observed in head and neck cancer (Xu *et al.* 1994).

How RAR β exerts its potent tumor-suppressive effects remains to be elucidated. A recent study demonstrated that RAR β can potentially inhibit AP-1 activity (Lin *et al.* 2000b) and induce apoptosis of various cancer cells. The proapoptotic effect of RAR β was implicated in the finding that the expression of RAR β in the developing mouse limb is highly restricted to the mesenchyme of the interdigital regions destined to undergo apoptosis (Dolle *et al.* 1989, Mendelsohn *et al.* 1991, Ruberte *et al.* 1991, Kochhar *et al.* 1993, Soprano *et al.* 1993a,b). In our previous study, we observed that *trans*-RA-induced apoptosis in ZR-75-1 breast cancer cells is mediated by RAR β (Liu *et al.* 1996). Inhibition of RAR β activity by the expression of RAR β anti-sense RNA reduced the number of apoptotic cells, whereas *trans*-RA-induced apoptosis was only observed in hormone-independent cells when RAR β was introduced and expressed in the cells (Liu *et al.* 1996).

The mechanism that causes loss of RAR β in prostate cancer is not clear. It is unlikely that lack of RAR β expression is due to structural abnormalities of the RAR β gene (Gebert *et al.* 1991), but possibly because of changes in transcription. Expression of RAR β is highly induced by *trans*-RA through a RARE (β RARE) present in its promoter (Hoffmann *et al.* 1990, Sucov *et al.* 1990, de The *et al.* 1990), which is activated by RAR/RXR heterodimers in response to retinoids (Zhang *et al.* 1992a). Vitamin A serum levels are lower in patients with prostate cancer (Reichman *et al.* 1990). In addition, prostate cancer tissues have five to eight times less *trans*-RA than normal prostate or benign prostate (Pasquali *et al.* 1996). Reduced levels of retinoids in prostate cancer tissue may contribute to loss of RAR β expression. Interestingly, RAR β cannot be induced by exogenous retinoids in androgen-independent prostate cancer cells, despite expression of RARs and RXRs in these cells (Sun *et al.* 1999b). Similar observations were also made in other cancer, such as lung cancer, cells which express RARs and RXRs, but fail to express RAR β in response to retinoids (Zhang *et al.* 1994). These observations argue against the involvement of reduced retinoid levels in inhibiting RAR β expression, and also demonstrate that expression of RARs and RXRs is not sufficient to render RAR β expression responsive to *trans*-RA. Thus, factors other than RARs and RXRs are required for the effect of *trans*-RA on inducing RAR β expression, and these may be lost in cancer cells. Recently, we found that expression of the orphan receptor COUP-TF is positively correlated with RAR β induction and growth inhibition by *trans*-RA in various cancer cell lines and it is underexpressed in many RAR β -negative cancer cell lines (Wu *et al.* 1997b, Lin *et al.* 2000a). Further studies demonstrated that COUP-TF is required for *trans*-RA to induce RAR β expression, growth inhibition and apoptosis in cancer cells (Lin *et al.* 2000a). The effect of COUP-TF is likely due to its transactivation of the RAR β promoter through its binding to a DR-8 element present in the promoter, resulting in enhanced interaction of RAR α with its co-activator CBP (Lin *et al.* 2000a). Thus, COUP-TF induces RAR β promoter transcription by acting as an accessory protein for RAR α to recruit its co-activator. Whether lack of COUP-TF expression is responsible for loss of RAR β in androgen-independent prostate cancer cells remains to be illustrated. Methylation of the RAR β promoter was recently reported to contribute to RAR β inactivity (Sirchia *et al.* 2000), suggesting a possibility of hypermethylation of the RAR β promoter in prostate cancer cells.

The anti-cancer effects of conventional retinoids appear to be limited to androgen-dependent prostate cancer cells, whereas the more aggressive, androgen-independent prostate cancer cells are refractory (Campbell *et al.* 1998). Loss of RAR β induction by *trans*-RA may be responsible for diminishment of *trans*-RA activities in androgen-independent prostate cancer cells. Induction of RAR β by classical retinoids,

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such as *trans*-RA, is mediated by activation of RAR/RXR heterodimers which bind to the β RARE (Zhang *et al.* 1992a). Unfortunately, this pathway appears to be impaired in androgen-independent prostate cancer cells. It is therefore important to identify alternative pathways that activate the RAR β promoter. Recent studies have demonstrated that RXR-selective retinoids represent promising agents for the prevention and treatment of cancer. 9-*cis* RA has demonstrated significant anti-proliferative and/or differentiating activity in *in vitro* models of breast cancer (Anzano *et al.* 1994, Rubin *et al.* 1994, Gottardis *et al.* 1996b), leukemia and lymphoma (Gottardis *et al.* 1996b), lung cancer (Guzey *et al.* 1998), and head and neck cancer (Giannini *et al.* 1997). Its activity was also observed in prostate cancer cells (Blutt *et al.* 1997, McCormick *et al.* 1999). Combination of 9-*cis* RA and 1,25-dihydroxyvitamin D3 synergistically inhibited the growth of LNCaP (Blutt *et al.* 1997, McCormick *et al.* 1999). McCormick *et al.* (1999) conducted a chemoprevention study to evaluate the activity of 9-*cis* RA as an inhibitor of prostate carcinogenesis in animals, and observed that continuous dietary administration of 9-*cis* RA before MNU administration reduced cancer incidence in the dorsolateral-anterior prostate. Similarly, the dosage levels of 9-*cis* RA reduced the incidence of cancer in all accessory sex glands (McCormick *et al.* 1999). RXR-selective retinoids were more effective than *trans*-RA at inhibiting mammary carcinogenesis in animals (Anzano *et al.* 1994), and RXR-selective retinoid LGD 1069 inhibited the growth of established breast tumors (Gottardis *et al.* 1996a, Bischoff *et al.* 1998).

How RXR ligands effectively inhibit the growth of cancer cells has not been established. Through its binding to RXR, RXR ligands may indirectly influence a wide range of functions, which are regulated by other nuclear receptors that heterodimerize with RXR (Zhang *et al.* 1992b, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). In our previous studies (Wu *et al.* 1997a), we observed that inhibition of cancer cell growth by RXR-selective retinoids was associated with induction of RAR β expression in estrogen-independent MDA-MB231 cells and lung cancer cells (Wu *et al.* 1997a), suggesting that induction of RAR β expression contributes to the growth-inhibitory effects of these retinoids. Furthermore, we observed that their effect on RAR β induction is in part mediated through TR3/RXR heterodimers which bind to the β RARE (Wu *et al.* 1997a). Thus, RXR ligands may exert their potent anti-cancer activity through inducing RAR β expression in cancer cells that are resistant to classical retinoids (Fig. 3). Thus, specific ligands for the RXR receptor may have significant activity as inhibitors of carcinogenesis in the prostate, whereas retinoids whose binding is limited to RAR may be inactive.

AHPN and its analogs: potent apoptotic inducers of prostate cancer cells

The sensitivity of prostate cancer cells to apoptosis-inducing effects of retinoids diminishes during the progression of

prostate tumors. Androgen-independent derivatives of LNCaP cells were more resistant than their parental androgen-dependent LNCaP cells to apoptotic effects of *trans*-RA. In addition, malignant prostate cancer cells showed resistance to radiotherapy and chemotherapy. This has been the major challenge in the therapy of prostate cancer. Thus, retinoids capable of inducing apoptosis of advanced malignant prostate cancer cells are expected to be suitable agents for prostate cancer treatment.

Recently, a new class of synthetic retinoids related to AHPN (also called CD437) (Bernard *et al.* 1992) has been found to potentially inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate carcinoma cells (Liang *et al.* 1999, Lu *et al.* 1999, Li *et al.* 2000, Sun *et al.* 2000). When the growth-inhibitory and apoptosis-inducing effects of *trans*-RA and AHPN were compared in androgen-dependent and -independent prostate cancer cell lines, AHPN significantly inhibited the growth and induced apoptosis of androgen-independent prostate cancer cell lines, while *trans*-RA had little effect on these cells (Sun *et al.* 2000). A synthetic retinoid, CD-271, which is related to AHPN and selectively activates the RAR γ subtype in a given context, also shows increased anti-proliferative activity against prostate cancer cells over *trans*-RA (Lu *et al.* 1999). Interestingly, AHPN was more effective in killing androgen-independent cells such as DU-145 and PC-3 than the androgen-dependent LNCaP cells (Sun *et al.* 2000). Thus, AHPN may be representative of a novel class of compounds suitable for treatment of androgen-independent prostate cancer. AHPN was also identified to be a potent apoptosis inducer in many different types of cancers, including lung (Sun *et al.* 1997, 1999c,d,e, Adachi *et al.* 1998b, Li *et al.* 1998), cervical (Oridate *et al.* 1997), ovarian (Langdon *et al.* 1998), melanoma (Schadendorf *et al.* 1995, 1996), leukemia (Hsu *et al.* 1997, Gianni & de The 1999, Mologni *et al.* 1999) and neuroblastoma (Meister *et al.* 1998). The apoptotic effect of AHPN is independent of retinoid receptor expression, indicating that its activity is not restricted by lack of RAR β in prostate cancer cells.

Orphan receptor TR3: a regulator of both survival and apoptosis of prostate cancer cells

AHPN-induced apoptosis may involve p53-dependent and -independent as well as caspase-dependent and -independent pathways (Adachi *et al.* 1998a, Fontana *et al.* 1998, Hsu *et al.* 1999, Marchetti *et al.* 1999, Zhang *et al.* 1999, Zhang 2000). Expression of a variety of apoptosis-associated genes, such as cJun, cFos, c-Myc, p21, Bcl-2, Bax, DR4, DR5 and Fas can be regulated by AHPN in a cell type-specific manner. Their role in AHPN-induced apoptosis remains to be determined. We have recently demonstrated that the expression of

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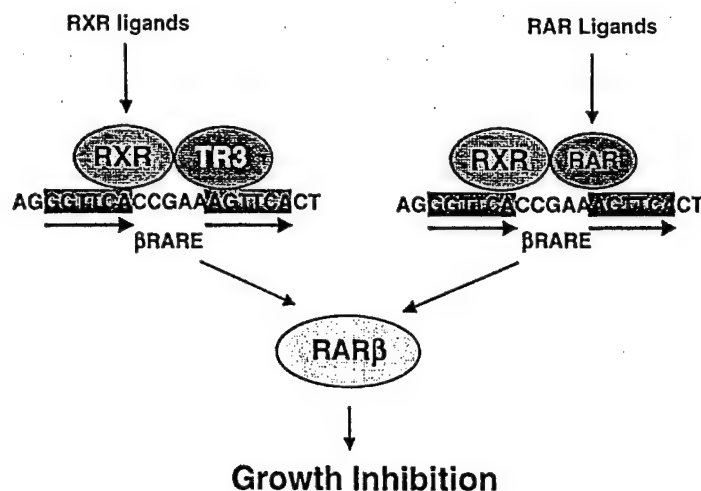


Figure 3 Signaling pathways for RAR β induction. β RARE in the RAR β promoter is essential for induction of RAR β by retinoids. The β RARE can be activated by RAR/RXR heterodimer in response to RAR ligands. Alternatively, it can be activated by RXR ligands through a TR3/RXR heterodimer that also binds to the β RARE.

TR3 is required for AHPN-induced apoptosis in human prostate cancer cells (Li *et al.* 2000). TR3 message was also highly induced by AHPN in LNCaP cells (Li *et al.* 2000). The apoptotic effect of the AHPN analog MM11453 was completely abolished in LNCaP cells stably expressing TR3 antisense RNA (Li *et al.* 2000).

TR3 (Chang & Kokontis 1988, Hazel *et al.* 1988, Milbrandt 1988) is an immediate early response gene whose expression is rapidly induced by a variety of growth stimuli, including growth factors, phorbol ester and cAMP-dependent pathways (Chang & Kokontis 1988, Hazel *et al.* 1988, Milbrandt 1988, Fahrner *et al.* 1990, Wilson *et al.* 1993, Crawford *et al.* 1995, Lim *et al.* 1995). It is also an orphan member of the steroid/thyroid/retinoid receptor superfamily (Zhang *et al.* 1992b, Kastner *et al.* 1995, Mangelsdorf & Evans 1995). Like other immediate early growth response genes, such as c-myc and c-jun, TR3 plays a role in controlling cell proliferation and mediating apoptosis (Bravo 1990, Herschman 1991). TR3 is rapidly induced during apoptosis in immature thymocytes and T-cell hybridomas (Liu *et al.* 1994, Woronicz *et al.* 1994). Overexpression of a dominant negative TR3 protein (Woronicz *et al.* 1994) or inhibition of TR3 expression by antisense TR3 inhibits apoptosis in thymocytes (Liu *et al.* 1994), whereas constitutive expression of TR3 results in massive apoptosis (Xue *et al.* 1997).

TR3 plays a critical role in regulating both proliferation and apoptosis of prostate cancer cells. Levels of TR3 are dramatically induced by androgen (Uemura & Chang 1998) and epidermal growth factor (Li *et al.* 2000) in LNCaP prostate cancer cells as well as by androgen ablation in the ventral prostate of animals (Uemura & Chang 1998). Interestingly, TR3 is also rapidly induced in LNCaP cells in

response to apoptotic stimuli, including AHPN (Li *et al.* 2000), calcium ionophore, etoposide (VP-16) (Uemura & Chang 1998, Li *et al.* 2000) and phorbol ester (Young *et al.* 1994, Li *et al.* 2000). Expression of TR3 antisense RNA significantly inhibits apoptosis induced by these agents (Li *et al.* 1998, Uemura & Chang 1998). Because of its potent effects in regulating cellular proliferation and apoptosis, TR3 may play a role in the development or progression of prostate cancer. In fact, TR3 is more highly expressed in prostate cancer areas than in adjacent normal or benign prostate hypertrophic tissue (Uemura & Chang 1998). TR3 is also highly expressed in lung cancer cell lines (Wu *et al.* 1997b). The role of TR3 in cancer development is further indicated by the finding that TR3 is involved in a chromosomal translocation identified in extra-skeletal myxoid chondrosarcoma (Labelle *et al.* 1995, 1999).

How TR3 exerts opposing biological activities is poorly understood. Similar to other members of the steroid/thyroid/retinoid receptor superfamily, it was believed that TR3 functioned in the nucleus as a transcriptional factor to regulate gene expression necessary to alter the cellular phenotype in response to various stimuli. TR3 response elements (NBRE or NurRE) have been identified (Wilson *et al.* 1991, Philips *et al.* 1997). In addition, TR3 can heterodimerize with RXR (Forman *et al.* 1995, Perlmann & Jansson 1995, Wu *et al.* 1997a) and COUP-TF (Wu *et al.* 1997b). The observations that over-expression of TR3 in cancer cells confers retinoid resistance by modulating transcriptional regulation of retinoids (Wu *et al.* 1997b) and that the TR3 fusion protein identified in extra-skeletal myxoid chondrosarcoma is about 270-fold more active than the native receptor in transactivation (Labelle *et al.* 1995, 1999) suggests that TR3 may mediate cell proliferation through its transcriptional regulation.

Much less is known about the mechanism by which TR3 functions to regulate apoptosis. TR3 might be involved in the apoptotic process by regulating expression of certain apoptosis-associated genes (Liu *et al.* 1994, Woronicz *et al.* 1994, 1995; Weih *et al.* 1996, Cheng *et al.* 1997). Unfortunately, no comprehensive characterization of its target genes has been achieved. By using a variety of approaches, we recently demonstrated that TR3-dependent apoptosis of LNCaP prostate cancer cells does not require its DNA binding and transactivation, but is associated with translocation of this protein from the nucleus to mitochondria, where it resides on the outer mitochondrial membrane and induces cytochrome c release (Li *et al.* 2000). These results reveal a novel mechanism by which a nuclear transcriptional factor translocates to mitochondria to initiate apoptosis (Fig. 4). Translocation of TR3 between the nucleus and the cytoplasm represents a new mechanism for cross-talk between different signaling pathways (Fig. 4). This exciting finding, together with the observations that TR3 is associated with cancer cell proliferation by acting as a nuclear transcriptional factor, demonstrates that the opposing biological activities of TR3 are regulated by its subcellular localization. These data suggest a new approach of eliminating prostate cancer cells by inducing cytoplasmic localization of TR3. AHPN analogs and other agents that specifically induce TR3 mitochondrial localization will not only induce apoptosis of prostate cancer cells mediated by TR3 mitochondrial action but also inhibit cancer cell proliferation induced by androgen or growth factors through nuclear action of TR3.

Related analogs may be potent inhibitors of androgen and growth factor action in prostate cancer cells.

Prospective

Induction of apoptosis is an effective way to eliminate cancer cells. The acquisition of resistance toward apoptosis during prostate tumor progression is perhaps the major obstacle in the treatment of prostate cancer. Retinoids inhibit the growth and induce apoptosis of prostate cancer cells *in vitro* and prevent prostate carcinogenesis in animals, suggesting that retinoids are promising agents for the prevention and treatment of human prostate cancer. However, the apoptotic effect of classical retinoids diminishes in androgen-independent prostate cancer cells, and clinical trials using conventional retinoids have not demonstrated significantly beneficial effects. Loss of RAR β may contribute to retinoid resistance in advanced prostate cancer cells. Alternative approaches to induce RAR β expression may render prostate cancer cells sensitive to apoptotic effects of retinoids. *In vitro* and animal studies have suggested that RXR ligands are effective inhibitors of prostate carcinogenesis and they are capable of inducing RAR β expression through alternative approaches, such as TR3/RXR heterodimers. Elucidation of their mechanisms of action will provide valuable information, allowing design and identification of a new generation of synthetic

retinoids that are likely to be more effective in the prevention and treatment of prostate cancer.

Synthetic retinoids related to AHPN effectively induce apoptosis of both androgen-dependent and -independent prostate cancer cells, indicating that these retinoids represent a new class of drugs that have therapeutic value for the treatment of prostate cancer. The clinical potential of this class of retinoids and their new generation needs to be explored.

Modern biology has suggested that cancer drug discovery based on molecular differences between tumor and normal cells is a new and feasible approach. With an improved understanding of apoptotic processes in prostate cancer cells, many potential new targets for therapy can be discovered. The illustration that orphan receptor TR3 mediates the apoptotic effect of AHPN analogs in prostate cancer cells suggests that TR3 is an ideal target for cancer drug development. Levels of TR3 are induced by androgen and growth factor in prostate cancer cells as well as by androgen ablation and may be necessary to support proliferation of prostate cancer cells. Thus, TR3 can mediate opposing biological activities, cell death and survival (Fig. 4). The unique property of TR3 provides an excellent opportunity to develop novel drugs targeted at TR3. Agents such as AHPN and its analogs that specifically induce mitochondrial localization of TR3 will convert TR3 from a cancer cell-promoting (adverse effect) to a cancer cell apoptosis-inducing (beneficial effect) molecule.

Cellular localization of TR3 defines its biological function. How TR3 is translocated from the nucleus to the cytoplasm and targets mitochondria in response to apoptotic stimuli is unclear. This information is essential for developing retinoids that induce mitochondrial localization of TR3. The fact that TR3 mitochondrial targeting is regulated by various stimuli, including TPA, calcium ionophore and growth factors (Li *et al.* 2000), which are known to act through membrane signaling pathways involving various kinases and phosphatases, suggests that phosphorylation of TR3 may play a crucial role in regulating TR3 subcellular activities.

The observation that TR3 can heterodimerize with RXR (Forman *et al.* 1995, Perlmann & Jansson 1995, Wu *et al.* 1997a) suggests that RXR and its ligands are likely involved in the regulation of TR3-dependent apoptotic pathways. This is supported by previous observations that RXR and its ligand 9-*cis*-RA inhibit activation-induced apoptosis of T-cells and thymocytes (Yang *et al.* 1993, 1995a,b, Bissonnette *et al.* 1995, Szondy *et al.* 1998), in which TR3 plays a role (Liu *et al.* 1994, Woronicz *et al.* 1994, 1995). RXR, through its heterodimerization with TR3, may be required for cytoplasmic localization of TR3 or for its mitochondrial targeting. Illustrating the molecular mechanisms by which RXR and its ligands regulate TR3-dependent apoptotic pathways in prostate cancer cells will provide additional modes to regulate apoptosis of prostate cancer cells and new treatment approaches for prostate cancer.

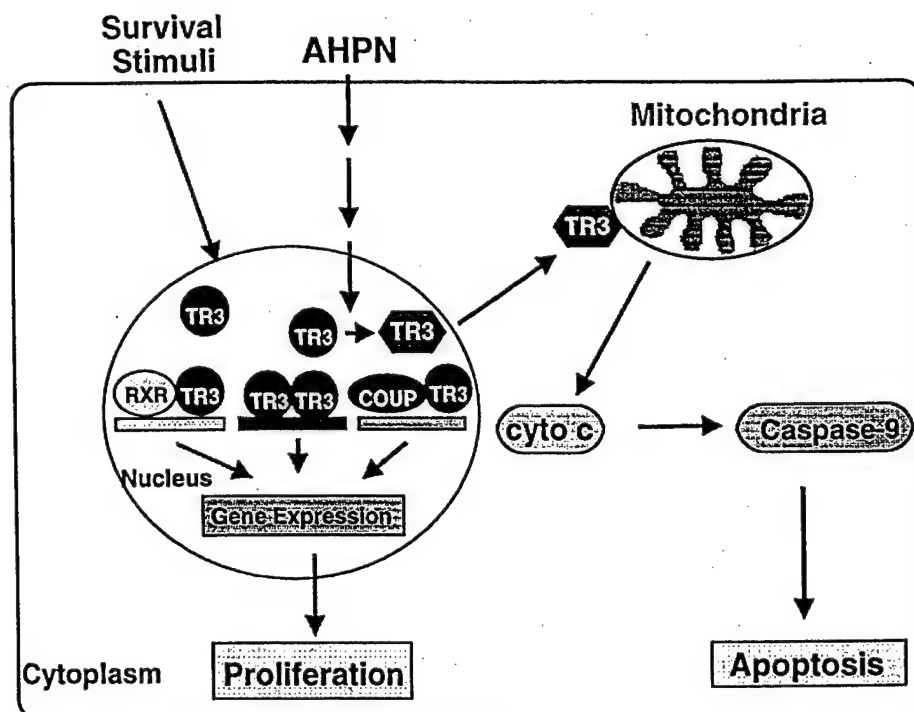


Figure 4 TR3-dependent cell survival and cell death pathways. TR3 induced by survival stimuli, such as growth factors, functions in the nucleus through either its homodimerization or heterodimerization with RXR or COUP-TF to regulate expression of genes involved in cell proliferation. In contrast, TR3 induced by death stimuli, including AHPN, may undergo a conformational change, which is required for its export to the cytoplasm, where it resides on mitochondria. On mitochondria, TR3 regulates mitochondrial activities, resulting in release of cytochrome c (cyto c) into the cytosol.

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References

- Aboself SR, Dahiya R, Narayan P & Cunha GR 1997 Effect of retinoic acid on prostatic development. *Prostate* 31 161-167.
- Adachi H, Adams A, Hughes FM, Zhang J, Cidlowski JA & Jetten AM 1998a Induction of apoptosis by the novel retinoid AHPN in human T-cell lymphoma cells involves caspase-dependent and independent pathways. *Cell Death and Differentiation* 5 973-983.
- Adachi H, Preston G, Harvat B, Dawson MI & Jetten AM 1998b Inhibition of cell proliferation and induction of apoptosis by the retinoid AHPN in human lung carcinoma cells. *American Journal of Respiratory Cell and Molecular Biology* 18 323-333.
- Adams JM & Cory S 1998 The Bcl-2 protein family: arbiters of cell survival. *Science* 281 1322-1326.
- Agus DB, Cordon-Cardo C, Fox W, Drobnjak M, Koff A, Golde DW & Scher HI 1999 Prostate cancer cell cycle regulators: response to androgen withdrawal and development of androgen independence. *Journal of the National Cancer Institute* 91 1869-1876.
- Alles AJ & Sulik KK 1990 Retinoic acid-induced spina bifida: evidence for a pathogenetic mechanism. *Development* 108 73-81.
- Alles AJ & Sulik KK 1992 Pathogenesis of retinoid-induced hindbrain malformations in an experimental model. *Clinical Dysmorphology* 1 187-200.
- Anzano MA, Byers SW, Smith JM, Peer CW, Mullen LT, Brown CC & Roberts AB 1994 Prevention of breast cancer in the rat with 9-cis-retinoic acid as a single agent and in combination with tamoxifen. *Cancer Research* 54 4614-4617.
- Berard J, Laboune F, Mukuna M, Masse S, Kothary R & Bradley WE 1996 Lung tumors in mice expressing an antisense RARbeta2 transgene. *FASEB Journal* 10 1091-1097.
- Bernard BA, Bernardon JM, Delescluse C, Martin B, Lenoir MC, Maignan J & Charpentier B 1992 Identification of synthetic retinoids with selectivity for human nuclear retinoic acid receptor gamma. *Biochemical and Biophysical Research Communications* 186 977-983.
- Bischoff ED, Gottardis MM, Moon TE, Heyman RA & Lamph WW 1998 Beyond tamoxifen: the retinoid X receptor-selective ligand LGD1069 (TARGRETIN) causes complete regression of mammary carcinoma. *Cancer Research* 58 479-484.
- Bissonnette RP, Brunner T, Lazarchik SB, Yoo NJ, Boehm MF, Green DR & Heyman RA 1995 9-cis retinoic acid inhibition of activation-induced apoptosis is mediated via regulation of fas

- ligand and requires retinoic acid receptor and retinoid X receptor activation. *Molecular and Cellular Biology* 15 5576-5585.
- Blutt SE, Allegretto EA, Pike JW & Weigel NL 1997 1,25-Dihydroxyvitamin D3 and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G1. *Endocrinology* 138 1491-1497.
- Bravo R 1990 Genes induced during the G0/G1 transition in mouse fibroblasts. *Seminars in Cancer Biology* 1 37-46.
- Bruckheimer EM & Kyprianou N 2000 Apoptosis in prostate carcinogenesis. A growth regulator and a therapeutic target. *Cell and Tissue Research* 301 153-162.
- Bruckheimer EM, Brisbay S, Johnson DJ, Gingrich JR, Greenberg N & McDonnell TJ 2000 Bcl-2 accelerates multistep prostate carcinogenesis *in vivo*. *Oncogene* 19 5251-5258.
- Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS & Anderson MJ 1999 Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96 857-868.
- Buttayan R, Ghafar MA & Shabsigh A 2000 The effects of androgen deprivation on the prostate gland: cell death mediated by vascular regression. *Current Opinion in Urology* 10 415-420.
- Campbell MJ, Park S, Uskokovic MR, Dawson MI & Koeffler HP 1998 Expression of retinoic acid receptor-beta sensitizes prostate cancer cells to growth inhibition mediated by combinations of retinoids and a 19-nor hexafluoride vitamin D3 analog. *Endocrinology* 139 1972-1980.
- Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E & Frisch S 1998 Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282 1318-1321.
- Chang C & Kokontis J 1988 Identification of a new member of the steroid receptor super-family by cloning and sequence analysis. *Biochemical and Biophysical Research Communications* 155 971-977.
- Chen JY, Penco S, Ostrowski J, Balaguer P, Pons M, Starrett JE & Reczek P 1995 RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *EMBO Journal* 14 1187-1197.
- Cheng LE, Chan FK, Cado D & Winoto A 1997 Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO Journal* 16 1865-1875.
- Chopra DP & Wilkoff LJ 1976 Inhibition and reversal by beta-retinoic acid of hyperplasia induced in cultured mouse prostate tissue by 3-methylcholanthrene or N-methyl-N'-nitro-N-nitrosoguanidine. *Journal of the National Cancer Institute* 56 583-589.
- Coffey RN, Watson RW & Fitzpatrick JM 2001 Signaling for the caspases: their role in prostate cell apoptosis. *Journal of Urology* 165 5-14.
- Crawford PA, Sadovsky Y, Woodson K, Lee SL & Milbrandt J 1995 Adrenocortical function and regulation of the steroid 21-hydroxylase gene in NGFI-B-deficient mice. *Molecular and Cellular Biology* 15 4331-4316.
- Culine S, Kramar A, Droz JP & Theodore C 1999 Phase II study of all-trans retinoic acid administered intermittently for hormone refractory prostate cancer. *Journal of Urology* 161 173-175.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y & Greenberg ME 1997 Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91 231-241.
- Denmeade SR, Lin XS & Isaacs JT 1996 Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* 28 251-265.
- Deveraux QL & Reed JC 1999 IAP family proteins -- suppressors of apoptosis. *Genes and Development* 13 239-252.
- Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C & Pandolfi PP 2001 Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. *Nature Genetics* 27 222-224.
- DiPaola RS & Aisner J 1999 Overcoming bcl-2- and p53-mediated resistance in prostate cancer. *Seminars in Oncology* 26 112-116.
- DiPaola RS, Weiss RE, Cummings KB, Kong FM, Jirtle RL, Anscher M & Gallo J 1997 Effect of 13-cis-retinoic acid and alpha-interferon on transforming growth factor beta1 in patients with rising prostate-specific antigen. *Clinical Cancer Research* 3 1999-2004.
- DiPaola RS, Rafi MM, Vyas V, Toppmeyer D, Rubin E, Patel J & Goodin S 1999 Phase I clinical and pharmacologic study of 13-cis-retinoic acid, interferon alfa, and paclitaxel in patients with prostate cancer and other advanced malignancies. *Journal of Clinical Oncology* 17 2213-2218.
- Dolle P, Ruberte E, Kastner P, Petkovich M, Stoner CM, Gudas LJ & Chambon P 1989 Differential expression of genes encoding alpha, beta and gamma retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature* 342 702-705.
- Du C, Fang M, Li Y, Li L & Wang X 2000 Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102 33-42.
- Fahrner TJ, Carroll SL & Milbrandt J 1990 The NGFI-B protein, an inducible member of the thyroid/steroid receptor family, is rapidly modified posttranslationally. *Molecular and Cellular Biology* 10 6454-6459.
- Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E & Graupner G 1994 A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature* 372 107-111.
- Ferrari N, Pfahl M & Levi G 1998 Retinoic acid receptor gamma1 (RARgamma1) levels control RARbeta2 expression in SK-N-BE2(c) neuroblastoma cells and regulate a differentiation-apoptosis switch. *Molecular and Cellular Biology* 18 6482-6492.
- Fisher DE 1994 Apoptosis in cancer therapy: crossing the threshold. *Cell* 78 539-542.
- Fontana JA, Sun RJ, Rishi AK, Dawson MI, Ordonez JV, Zhang Y & Tschang SH 1998 Overexpression of bcl-2 or bcl-XL fails to inhibit apoptosis mediated by a novel retinoid. *Oncology Research* 10 313-324.
- Forman BM, Umesono K, Chen J & Evans RM 1995 Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* 81 541-550.
- Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ & Neel BG 1991 High frequency of retinoic acid receptor beta abnormalities in human lung cancer. *Oncogene* 6 1859-1868.
- Gianni M & de The H 1999 In acute promyelocytic leukemia NB4 cells, the synthetic retinoid CD437 induces contemporaneously apoptosis, a caspase-3-mediated degradation of PML/RARalpha protein and the PML retargeting on PML-nuclear bodies. *Leukemia* 13 739-749.
- Giannini F, Maestro R, Vukosavljevic T, Pomponi F & Boiocchi M 1997 All-trans, 13-cis and 9-cis retinoic acids induce a fully reversible growth inhibition in HNSCC cell lines: implications for *in vivo* retinoic acid use. *International Journal of Cancer* 70 194-200.

- Gleave M, Tolcher A, Miyake H, Nelson C, Brown B, Beraldi E & Goldie J 1999 Progression to androgen independence is delayed by adjuvant treatment with antisense Bcl-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model. *Clinical Cancer Research* 5 2891-2898.
- Goossens K, Esquenet M, Swinnen JV, Manin M, Rombauts W & Verhoeven G 1999 Androgens decrease and retinoids increase the expression of insulin-like growth factor-binding protein-3 in LNCaP prostatic adenocarcinoma cells. *Molecular and Cellular Endocrinology* 155 9-18.
- Gottardis MM, Bischoff ED, Shirley MA, Wagoner MA, Lamph WW & Heyman RA 1996a Chemoprevention of mammary carcinoma by LGD1069 (Targretin): an RXR-selective ligand. *Cancer Research* 56 5566-5570.
- Gottardis MM, Lamph WW, Shalinsky DR, Wellstein A & Heyman RA 1996b The efficacy of 9-cis retinoic acid in experimental models of cancer. *Breast Cancer Research and Treatment* 38 85-96.
- Guzey M, Demirpence E, Criss W & DeLuca HF 1998 Effects of retinoic acid (all-trans and 9-cis) on tumor progression in small-cell lung carcinoma. *Biochemical and Biophysical Research Communications* 242 369-375.
- Hazel TG, Nathans D & Lau LF 1988 A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *PNAS* 85 8444-8448.
- Herschman HR 1991 Primary response genes induced by growth factors and tumor promoters. *Annual Review of Biochemistry* 60 281-319.
- Hoffman AD, Engelstein D, Bogenrieder T, Papandreou CN, Steckelman E, Dave A & Motzer RJ 1996 Expression of retinoic acid receptor beta in human renal cell carcinomas correlates with sensitivity to the antiproliferative effects of 13-cis-retinoic acid. *Clinical Cancer Research* 2 1077-1082.
- Hoffmann B, Lehmann JM, Zhang XK, Hermann T, Husmann M, Graupner G & Pfahl M 1990 A retinoic acid receptor-specific element controls the retinoic acid receptor-beta promoter. *Molecular Endocrinology* 4 1727-1736.
- Howell SB 2000 Resistance to apoptosis in prostate cancer cells. *Molecular Urology* 4 225-229; discussion 231.
- Hsu CA, Rishi AK, Su-Li X, Gerald TM, Dawson MI, Schiffer C & Reichert U 1997 Retinoid induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. *Blood* 89 4470-4479.
- Hsu SL, Yin SC, Liu MC, Reichert U & Ho WL 1999 Involvement of cyclin-dependent kinase activities in CD437-induced apoptosis. *Experimental Cell Research* 252 332-341.
- Ittmann MM 1998 Chromosome 10 alterations in prostate adenocarcinoma (review). *Oncology Reports* 5 1329-1335.
- Kaiser A, Herbst H, Fisher G, Koenigsmann M, Berdel WE, Riecken EO & Rosewicz S 1997 Retinoic acid receptor beta regulates growth and differentiation in human pancreatic carcinoma cells. *Gastroenterology* 113 920-929.
- Kastner P, Mark M & Chambon P 1995 Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83 859-869.
- Katagiri Y, Takeda K, Yu ZX, Ferrans VJ, Ozato K & Guroff G 2000 Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B. *Nature Cell Biology* 2 435-440.
- Kelly WK, Osman I, Reuter VE, Curley T, Heston WD, Nanus DM & Scher HI 2000 The development of biologic end points in patients treated with differentiation agents: an experience of retinoids in prostate cancer. *Clinical Cancer Research* 6 838-846.
- Kochhar DM, Jiang H, Harnish DC & Soprano DR 1993 Evidence that retinoic acid-induced apoptosis in the mouse limb bud core mesenchymal cells is gene-mediated. *Progress in Clinical and Biological Research* 315 815-825.
- Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL & Burgering BM 1999 Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* 398 630-634.
- Koshiuka K, Elstner E, Williamson E, Said JW, Tada Y & Koeffler HP 2000 Novel therapeutic approach: organic arsenical melarsoprol alone or with all-trans-retinoic acid markedly inhibit growth of human breast and prostate cancer cells *in vitro* and *in vivo*. *British Journal of Cancer* 82 452-458.
- Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS *et al.* 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 104 719-730.
- Labelle Y, Zucman J, Stenman G, Kindblom LG, Knight J, Turc-Carel C & Dockhorn-Dworniczak B 1995 Oncogenic conversion of a novel orphan nuclear receptor by chromosome translocation. *Human Molecular Genetics* 4 2219-2226.
- Labelle Y, Bussieres J, Courjal F & Goldring MB 1999 The EWS/TEC fusion protein encoded by the t(9;22) chromosomal translocation in human chondrosarcomas is a highly potent transcriptional activator. *Oncogene* 18 3303-3308.
- Langdon SP, Rabiasz GJ, Ritchie AA, Reichert U, Buchan P, Miller WR & Smyth JF 1998 Growth-inhibitory effects of the synthetic retinoid CD437 against ovarian carcinoma models *in vitro* and *in vivo*. *Cancer Chemotherapy and Pharmacology* 42 429-432.
- Lasnitzki I & Goodman DS 1974 Inhibition of the effects of methylcholanthrene on mouse prostate in organ culture by vitamin A and its analogs. *Cancer Research* 34 1564-1571.
- Leung S, Miyake H, Zellweger T, Tolcher A & Gleave ME 2001 Synergistic chemosensitization and inhibition of progression to androgen independence by antisense Bcl-2 oligodeoxynucleotide and paclitaxel in the LNCaP prostate tumor model. *International Journal of Cancer* 91 846-850.
- Li C & Wan YJ 1998 Differentiation and antiproliferation effects of retinoic acid receptor beta in hepatoma cells. *Cancer Letters* 124 205-211.
- Li H, Kolluri SK, Gu J, Dawson MI, Cao X, Hobbs PD & Lin B 2000 Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3 (see comments) (comment). *Science* 289 1159-1164.
- Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI & Puc J 1997 PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275 1943-1947.
- Li JJ, Dong Z, Dawson MI & Colburn NH 1996 Inhibition of tumor promoter-induced transformation by retinoids that transrepress AP-1 without transactivating retinoic acid response element. *Cancer Research* 56 483-489.
- Li X, Marani M, Mannucci R, Kinsey B, Andriani F, Nicoletti I & Denner L 2001 Overexpression of BCL-X(L) underlies the molecular basis for resistance to staurosporine-induced apoptosis in PC-3 cells. *Cancer Research* 61 1699-1706.
- Li XS, Shao ZM, Sheikh MS, Eiseman JL, Sentz D, Jetten AM & Chen JC 1995 Retinoic acid nuclear receptor beta inhibits breast

- carcinoma anchorage independent growth. *Journal of Cellular Physiology* 165 449-458.
- Li Y, Lin B, Agadir A, Liu R, Dawson MI, Reed JC & Fontana JA 1998 Molecular determinants of AHPN (CD437)-induced growth arrest and apoptosis in human lung cancer cell lines. *Molecular and Cellular Biology* 18 4719-4731.
- Liang JY, Fontana JA, Rao JN, Ordonez JV, Dawson MI, Shroot B & Wilber JF 1999 Synthetic retinoid CD437 induces S-phase arrest and apoptosis in human prostate cancer cells LNCaP and PC-3. *Prostate* 38 228-236.
- Lim RW, Zhu CY & Stringer B 1995 Differential regulation of primary response gene expression in skeletal muscle cells through multiple signal transduction pathways. *Biochimica et Biophysica Acta* 1266 91-100.
- Lin B, Chen GQ, Xiao D, Kolluri SK, Cao X, Su H & Zhang XK 2000a Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition, and apoptosis by retinoic acid in cancer cells. *Molecular and Cellular Biology* 20 957-970.
- Lin F, Xiao D, Kolluri SK & Zhang X 2000b Unique anti-activator protein-1 activity of retinoic acid receptor beta. *Cancer Research* 60 3271-3280.
- Liu B, Lee HY, Weinzier SA, Powell DR, Clifford JL, Kurie JM & Cohen P 2000 Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor-alpha regulate transcriptional signaling and apoptosis. *Journal of Biological Chemistry* 275 33607-33613.
- Liu Y, Lee MO, Wang HG, Li Y, Hashimoto Y, Klaus M & Reed JC 1996 Retinoic acid receptor beta mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Molecular and Cellular Biology* 16 1138-1149.
- Liu ZG, Smith SW, McLaughlin KA, Schwartz LM & Osborne BA 1994 Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* 367 281-284.
- Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ & Hong WK 1995 Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. *New England Journal of Medicine* 332 1405-1410.
- Lotan Y, Xu XC, Shalev M, Lotan R, Williams R, Wheeler TM & Thompson TC 2000 Differential expression of nuclear retinoid receptors in normal and malignant prostates. *Journal of Clinical Oncology* 18 116-121.
- Lu XP, Fanjul A, Picard N, Shroot B & Pfahl M 1999 A selective retinoid with high activity against an androgen-resistant prostate cancer cell type. *International Journal of Cancer* 80 272-278.
- McCormick DL, Rao KV, Steele VE, Lubet RA, Kelloff GJ & Bosland MC 1999 Chemoprevention of rat prostate carcinogenesis by 9-cis-retinoic acid. *Cancer Research* 59 521-524.
- Mangelsdorf DJ & Evans RM 1995 The RXR heterodimers and orphan receptors. *Cell* 83 841-850.
- Marchetti P, Zamzami N, Joseph B, Schraen-Maschke S, Mereau-Richard C, Costantini P & Metivier D 1999 The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. *Cancer Research* 59 6257-6266.
- Medema RH, Kops GJ, Bos JL & Burgering BM 2000 AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* 404 782-787.
- Meister B, Fink FM, Hittmair A, Marth C & Widschwendter M 1998 Antiproliferative activity and apoptosis induced by retinoic acid receptor-gamma selectively binding retinoids in neuroblastoma. *Anticancer Research* 18 1777-1786.
- Mendelsohn C, Ruberte E, LeMeur M, Morriss-Kay G & Chambon P 1991 Developmental analysis of the retinoic acid-inducible RAR-beta 2 promoter in transgenic animals. *Development* 113 723-734.
- Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M & Barone MV 2000 Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *EMBO Journal* 19 5406-5417.
- Milbrandt J 1988 Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* 1 183-188.
- Mologni L, Ponzanelli I, Bresciani F, Sardiello G, Bergamaschi D, Gianni M & Reichert U 1999 The novel synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) causes apoptosis in acute promyelocytic leukemia cells through rapid activation of caspases. *Blood* 93 1045-1061.
- Montironi R, Pomante R, Diamanti L & Magi-Galluzzi C 1998 Apoptosis in prostatic adenocarcinoma following complete androgen ablation. *Urology International* 60 25-29; discussion 30.
- Nagy L, Thomazy VA, Heyman RA & Davies PJ 1998 Retinoid-induced apoptosis in normal and neoplastic tissues. *Cell Death Differentiation* 5 11-19.
- Nehme A, Varadarajan P, Sellakumar G, Gerhold M, Niedner H, Zhang Q & Lin X 2001 Modulation of docetaxel-induced apoptosis and cell cycle arrest by all-trans retinoic acid in prostate cancer cells. *British Journal of Cancer* 84 1571-1576.
- Nervi C, Vollberg TM, George MD, Zelent A, Chambon P & Jetten AM 1991 Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Experimental Cell Research* 195 163-170.
- Nunez G, Benedict MA, Hu Y & Inohara N 1998 Caspases: the proteases of the apoptotic pathway. *Oncogene* 17 3237-3245.
- Oridate N, Higuchi M, Suzuki S, Shroot B, Hong WK & Lotan R 1997 Rapid induction of apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-adamantyl)hydroxyphenyl]-2-naphthalene carboxylic acid (CD437). *International Journal of Cancer* 70 484-487.
- Pasquali D, Thaller C & Eichele G 1996 Abnormal level of retinoic acid in prostate cancer tissues. *Journal of Clinical Endocrinology and Metabolism* 81 2186-2191.
- Pasquali D, Rossi V, Prezioso D, Gentile V, Colantuoni V, Lotti T & Bellastella A 1999 Changes in tissue transglutaminase activity and expression during retinoic acid-induced growth arrest and apoptosis in primary cultures of human epithelial prostate cells. *Journal of Clinical Endocrinology and Metabolism* 84 1463-1469.
- Perlmann T & Jansson L 1995 A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes and Development* 9 769-782.
- Pfahl M 1993 Nuclear receptor/AP-1 interaction. *Endocrine Reviews* 14 651-658.
- Philips A, Lesage S, Gingras R, Maira MH, Gauthier Y, Hugo P & Drouin J 1997 Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. *Molecular and Cellular Biology* 17 5946-5951.
- Pili R, Kruszewski MP, Hager BW, Lantz J & Carducci MA 2001 Combination of phenylbutyrate and 13-cis retinoic acid inhibits

- prostate tumor growth and angiogenesis. *Cancer Research* 61 1477-1485.
- Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC & Perucho M 1997 Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 275 967-969.
- Reed JC 1998 Bcl-2 family proteins. *Oncogene* 17 3225-3236.
- Reichman ME, Hayes RB, Ziegler RG, Schatzkin A, Taylor PR, Kahle LL & Fraumeni JF Jr 1990 Serum vitamin A and subsequent development of prostate cancer in the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *Cancer Research* 50 2311-2315.
- Reuter VE 1997 Pathological changes in benign and malignant prostatic tissue following androgen deprivation therapy. *Urology* 49 16-22.
- Richter F, Huang HF, Li MT, Danielpour D, Wang SL & Irwin RJ Jr 1999 Retinoid and androgen regulation of cell growth, epidermal growth factor and retinoic acid receptors in normal and carcinoma rat prostate cells. *Molecular and Cellular Endocrinology* 153 29-38.
- Ruberte E, Dolle P, Chambon P & Morriss-Kay G 1991 Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* 111 45-60.
- Rubin M, Fenig E, Rosenauer A, Menendez-Botet C, Achkar C, Bentel JM & Yahalom J 1994 9-Cis retinoic acid inhibits growth of breast cancer cells and down-regulates estrogen receptor RNA and protein. *Cancer Research* 54 6549-6556.
- Schadendorf D, Worm M, Jurgovsky K, Dippel E, Reichert U & Czarnetzki BM 1995 Effects of various synthetic retinoids on proliferation and immunophenotype of human melanoma cells *in vitro*. *Recent Results in Cancer Research* 139 183-193.
- Schadendorf D, Kern MA, Artuc M, Pahl HL, Rosenbach T, Fichtner I & Nurnberg W 1996 Treatment of melanoma cells with the synthetic retinoid CD437 induces apoptosis via activation of AP-1 *in vitro*, and causes growth inhibition in xenografts *in vivo*. *Journal of Cell Biology* 135 1889-1898.
- Scheller K, Sekeris CE, Krohne G, Hock R, Hansen IA & Scheer U 2000 Localization of glucocorticoid hormone receptors in mitochondria of human cells. *European Journal of Cell Biology* 79 299-307.
- Shalev M, Thompson TC, Frolov A, Lippman SM, Hong WK, Fritsche H & Kadmon D 2000 Effect of 13-cis-retinoic acid on serum prostate-specific antigen levels in patients with recurrent prostate cancer after radical prostatectomy. *Clinical Cancer Research* 6 3845-3849.
- Shi L, Kraut RP, Aebersold R & Greenberg AH 1992 A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *Journal of Experimental Medicine* 175 553-566.
- Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW & Liao JK 2000 Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407 538-541.
- Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S & Sacchi N 2000 Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. *Oncogene* 19 1556-1563.
- Soprano DR, Harnish DC, Soprano KJ, Kochhar DM & Jiang H 1993a Correlations of RAR isoforms and cellular retinoid-binding proteins mRNA levels with retinoid-induced teratogenesis. *Journal of Nutrition* 123 367-371.
- Soprano DR, Tairis N, Gyda M 3rd, Harnish DC, Jiang H, Soprano KJ & Kochhar DM 1993b Induction of RAR-beta 2 gene expression in embryos and RAR-beta 2 transactivation by the synthetic retinoid Ro 13-6307 correlates with its high teratogenic potency. *Toxicology and Applied Pharmacology* 122 159-163.
- Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T & Ruland J 1998 Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 95 29-39.
- Steller H 1995 Mechanisms and genes of cellular suicide. *Science* 267 1445-1449.
- Sucov HM, Murakami KK & Evans RM 1990 Characterization of an autoregulated response element in the mouse retinoic acid receptor type beta gene. *PNAS* 87 5392-5396.
- Sulik KK & Dehart DB 1988 Retinoic-acid-induced limb malformations resulting from apical ectodermal ridge cell death. *Teratology* 37 527-537.
- Sulik KK, Cook CS & Webster WS 1988 Teratogens and craniofacial malformations: relationships to cell death. *Development* 103 213-231.
- Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J & Gavrilova N 1999a PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5-trisphosphate and Akt/protein kinase B signaling pathway. *PNAS* 96 6199-6204.
- Sun SY, Yue P, Shroot B, Hong WK & Lotan R 1997 Induction of apoptosis in human non-small cell lung carcinoma cells by the novel synthetic retinoid CD437. *Journal of Cellular Physiology* 173 279-284.
- Sun SY, Yue P & Lotan R 1999b Induction of apoptosis by N-(4-hydroxyphenyl)retinamide and its association with reactive oxygen species, nuclear retinoic acid receptors, and apoptosis-related genes in human prostate carcinoma cells. *Molecular Pharmacology* 55 403-410.
- Sun SY, Yue P, Shroot B, Hong WK & Lotan R 1999c Implication of c-Myc in apoptosis induced by the retinoid CD437 in human lung carcinoma cells. *Oncogene* 18 3894-3901.
- Sun SY, Yue P, Wu GS, El-Deiry WS, Shroot B, Hong WK & Lotan R 1999d Implication of p53 in growth arrest and apoptosis induced by the synthetic retinoid CD437 in human lung cancer cells. *Cancer Research* 59 2829-2833.
- Sun SY, Yue P, Wu GS, El-Deiry WS, Shroot B, Hong WK & Lotan R 1999e Mechanisms of apoptosis induced by the synthetic retinoid CD437 in human non-small cell lung carcinoma cells. *Oncogene* 18 2357-2365.
- Sun SY, Yue P & Lotan R 2000 Implication of multiple mechanisms in apoptosis induced by the synthetic retinoid CD437 in human prostate carcinoma cells. *Oncogene* 19 4513-4522.
- Szondy Z, Reichert U & Fesus L 1998 Retinoic acids regulate apoptosis of T lymphocytes through an interplay between RAR and RXR receptors (see comments). *Cell Death Differentiation* 5 4-10.
- Szostak MJ & Kyprianou N 2000 Radiation-induced apoptosis: predictive and therapeutic significance in radiotherapy of prostate cancer (review). *Oncology Reports* 7 699-706.
- Tanabe T 2000 Effects of N-(4-hydroxyphenyl) retinamide on urokinase-type plasminogen activator and plasminogen activator inhibitor-1 in prostate adenocarcinoma cell lines. *Hiroshima Journal of Medical Science(s)* 49 67-72.

- Thaller C, Shalev M, Frolov A, Eichele G, Thompson TC, Williams RH & Dilliogluligil O 2000 Fenretinide therapy in prostate cancer: effects on tissue and serum retinoid concentration. *Journal of Clinical Oncology* 18 3804-3808.
- de The H, Vivanco-Ruiz MM, Tiollais P, Stunnenberg H & Dejean A 1990 Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. *Nature* 343 177-180.
- Thompson CB 1995 Apoptosis in the pathogenesis and treatment of disease. *Science* 267 1456-1462.
- Thornberry NA & Lazebnik Y 1998 Caspases: enemies within. *Science* 281 1312-1316.
- Uemura H & Chang C 1998 Antisense TR3 orphan receptor can increase prostate cancer cell viability with etoposide treatment. *Endocrinology* 139 2329-2334.
- Urban D, Myers R, Manne U, Weiss H, Mohler J, Perkins D & Markiewicz M 1999 Evaluation of biomarker modulation by fenretinide in prostate cancer patients. *European Urology* 35 429-438.
- Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE & Moritz RL 2000 Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102 43-53.
- Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ & Trapman J 1998 Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. *Cancer Research* 58 2720-2723.
- Wang XD, Liu C, Bronson RT, Smith DE, Krinsky NI & Russell M 1999 Retinoid signaling and activator protein-1 expression in ferrets given beta-carotene supplements and exposed to tobacco smoke (see comments). *Journal of the National Cancer Institute* 91 60-66.
- Webber MM, Bello-DeOcampo D, Quader S, Deocampo ND, Metcalfe WS & Sharp RM 1999 Modulation of the malignant phenotype of human prostate cancer cells by N-(4-hydroxyphenyl)retinamide (4-HPR). *Clinical and Experimental Metastasis* 17 255-263.
- Weih F, Ryseck RP, Chen L & Bravo R 1996 Apoptosis of nur77/N10-transgenic thymocytes involves the Fas/Fas ligand pathway. *PNAS* 93 5533-5538.
- Whang YE, Wu X, Suzuki H, Reiter RE, Tran C, Vessella RL & Said JW 1998 Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *PNAS* 95 5246-5250.
- White E 1996 Life, death, and the pursuit of apoptosis. *Genes and Development* 10 1-15.
- Wilson TE, Fahrner TJ, Johnston M & Milbrandt J 1991 Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science* 252 1296-1300.
- Wilson TE, Fahrner TJ & Milbrandt J 1993 The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Molecular and Cellular Biology* 13 5794-5804.
- Woronicz JD, Calnan B, Ngo V & Winoto A 1994 Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* 367 277-281.
- Woronicz JD, Lina A, Calnan BJ, Szychowski S, Cheng L & Winoto A 1995 Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis. *Molecular and Cellular Biology* 15 6364-6376.
- Wu Q, Dawson MI, Zheng Y, Hobbs PD, Agadir A, Jong L & Li Y 1997a Inhibition of trans-retinoic acid-resistant human breast cancer cell growth by retinoid X receptor-selective retinoids. *Molecular and Cellular Biology* 17 6598-6608.
- Wu Q, Li Y, Liu R, Agadir A, Lee MO, Liu Y & Zhang X 1997b Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization. *EMBO Journal* 16 1656-1669.
- Wu X, Senechal K, Neshat MS, Whang YE & Sawyers CL 1998 The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *PNAS* 95 15587-15591.
- Xu L, Glass CK & Rosenfeld MG 1999 Coactivator and corepressor complexes in nuclear receptor function. *Current Opinion in Genetic Development* 9 140-147.
- Xu XC, Ro JY, Lee JS, Shin DM, Hong WK & Lotan R 1994 Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. *Cancer Research* 54 3580-3587.
- Xu XC, Sneige N, Liu X, Nandagiri R, Lee JJ, Lukmanji F & Hortobagyi G 1997a Progressive decrease in nuclear retinoic acid receptor beta messenger RNA level during breast carcinogenesis. *Cancer Research* 57 4992-4996.
- Xu XC, Sozzi G, Lee JS, Lee JJ, Pastorino U, Pilotti S & Kurie JM 1997b Suppression of retinoic acid receptor beta in non-small-cell lung cancer in vivo: implications for lung cancer development (see comments). *Journal of the National Cancer Institute* 89 624-629.
- Xue Y, Chomez P, Castanos-Velez E, Biberfeld P, Perlmann T & Jondal M 1997 Positive and negative thymic selection in T cell receptor-transgenic mice correlate with Nur77 mRNA expression (published erratum appears in *European Journal of Immunology* (1997) 27 2748). *European Journal of Immunology* 27 2048-2056.
- Yang Y, Vacchio MS & Ashwell JD 1993 9-cis-retinoic acid inhibits activation-driven T-cell apoptosis: implications for retinoid X receptor involvement in thymocyte development. *PNAS* 90 6170-6174.
- Yang Y, Bailey J, Vacchio MS, Yarchoan R & Ashwell JD 1995a Retinoic acid inhibition of ex vivo human immunodeficiency virus-associated apoptosis of peripheral blood cells. *PNAS* 92 3051-3055.
- Yang Y, Minucci S, Ozato K, Heyman RA & Ashwell JD 1995b Efficient inhibition of activation-induced Fas ligand up-regulation and T cell apoptosis by retinoids requires occupancy of both retinoid X receptors and retinoic acid receptors. *Journal of Biological Chemistry* 270 18672-18677.
- Young CY, Murtha PE & Zhang J 1994 Tumor-promoting phorbol ester-induced cell death and gene expression in a human prostate adenocarcinoma cell line. *Oncology Research* 6 203-210.
- Zhang XK & Pfahl M 1993 Hetero- and homodimeric receptors in thyroid hormone and vitamin A action. *Receptor* 3 183-191.
- Zhang XK, Hoffmann B, Tran PB, Graupner G & Pfahl M 1992a Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* 355 441-446.
- Zhang XK, Lehmann J, Hoffmann B, Dawson MI, Cameron J, Graupner G & Hermann T 1992b Homodimer formation of

- retinoid X receptor induced by 9-cis retinoic acid. *Nature* 358 587-591.
- Zhang XK, Liu Y, Lee MO & Pfahl M 1994 A specific defect in the retinoic acid response associated with human lung cancer cell lines. *Cancer Research* 54 5663-5669.

- Zhang Y, Huang Y, Rishi AK, Sheikh MS, Shroot B, Reichert U & Dawson M 1999 Activation of the p38 and JNK/SAPK mitogen-activated protein kinase pathways during apoptosis is mediated by a novel retinoid. *Experimental Cell Research* 247 233-240.